Isolation and Characterization of an Epithelial-specific Receptor Tyrosine Kinase from an Ovarian Cancer Cell Line

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Abstract
A protein receptor tyrosine kinase (RTK) 6 has been isolated from a complementary DNA library of SKOV-3, an epithelial ovarian cancer cell line, using a polymerase chain reaction (PCR)-mediated approach. The primary structure of the predicted amino acid sequence of the protein shows a novel NH₂-terminal region which has homology to a factor VIII-like domain. The junctional domain region is proline and glycine rich and is the longest for any known receptor kinase. The COOH-terminal catalytic domain has all of the canonical sequence motifs of a receptor tyrosine kinase with homology to the TRK-H2 protein (49%). A single transcript of 4.5 kilobases is expressed at low levels in heart, placenta, lung, liver, muscle, kidney, and pancreas, with high levels of expression in the brain. Ribonuclease protection assay showed a varying level of expression of message in a panel of eight ovarian cancer cell lines compared to placenta. In situ hybridization analysis demonstrated localization of mRNA in the epithelial cells of the ovary, kidney, small bowel, lung, thymus, and brain. There was a lower level of message in normal, benign, and borderline tumors of the ovary compared to malignant tumors of the ovary. Polyclonal antisera raised against a COOH-terminal synthetic peptide recognize a M1, 140,000 protein in ovarian cancer cells, which autophosphorylates in an in vitro kinase assay.

Introduction
Protein tyrosine kinases play an important role in cellular metabolism as key components of signal transduction pathways. They are involved in cellular growth, differentiation, and development (1–3). They are broadly categorized into receptor and nonreceptor tyrosine kinases. RTKs1 have been shown to be receptors of several growth factors and as products of the oncogenes of several acute transforming retroviruses. There are at least 10 classes of RTKs described. These include the families of epidermal growth factor receptor, the insulin receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor, eph receptors, trk receptors, and the recently described tie and tek receptors (4, 5). The primary structure of all RTKs show a variable extracellular domain, transmembrane domain, and a conserved catalytic domain (6). In several cases, the transduction of signals across the membrane has been shown to be due to ligand-induced receptor dimerization or oligomerization and subsequent intracellular receptor autophosphorylation. This autophosphorylation has now been shown to lead to an association with adapter proteins (Grb2/Shc) through their Src homology 2 (SH2) and Src homology 3 domains. This in turn is associated with the mammalian son-of-sevenless gene product, which in turn activates Ras for the human epidermal growth factor receptor and insulin receptor (7, 8, 9). The subsequent pathway involves Raf and the MAP kinases, ultimately leading to transcription and DNA synthesis. It is possible that disruption anywhere in this pathway can lead to abnormal cellular proliferation. Additionally, overexpression of several different receptor kinases can induce transformation in the appropriate cell type.

There is clear clinical relevance for at least two receptor tyrosine kinases of the type I class. Epidermal growth factor receptor and c-erbB2 have been shown to be overexpressed in tumors from several cancers. This has been shown to be an adverse prognostic factor which correlates with poor survival of patients (10, 11). In epithelial ovarian cancer, it has been demonstrated that c-erbB2, a type I receptor tyrosine kinase, is overexpressed in 20–30% of tumors (10, 11). The survival of patients with tumours overexpressing c-erbB2 is relatively poor.

There are several approaches which have been defined in the literature to isolate new members of the protein kinase family. The most successful strategy has been that based on the PCR, leading to the isolation of several new members in a relatively short time (12). This is based on the strongly conserved catalytic domain amongst all protein kinases (6). In order to isolate potentially significant kinases in ovarian cancer, a strategy using PCR and degenerate oligonucleotide primers for two invariant motifs within the conserved catalytic domain was used. This paper reports the isolation, expression, and characterization of a new receptor tyrosine kinase (RTK6) of the type II class. The extracellular domain of this RTK is unusual and has a motif which has homology with a repeat domain found in factor VIII, factor V, milk fat globule membrane protein, A5 antigen, and discoidin (13–18). It is widely expressed in epithelial cells.

Results
Isolation of RTK6 cDNA Clones. The cDNA for RTK6 was isolated during a search for tyrosine kinases expressed in ovarian cells. The amplification of sequences between two

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3 The abbreviations used are: RTK, receptor tyrosine kinase; PCR, polymerase chain reaction; cDNA, complementary DNA; bp, base pair(s); poly(A)+, polyadenylated; kb, kilobases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; NGF, nerve growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
conserved motifs within the catalytic domain of all kinases by PCR from an epithelial ovarian cancer cell line cDNA library (SKOV-3; Ref. 19) identified two potentially novel sequences (see "Material and Methods"). The first sequence, RTK6, approximately 400 bp, was used to screen the cDNA library of SKOV-3. Two partial overlapping clones (6D and 6D1F) were obtained. Additional 5' sequence was obtained from a placent cDNA library by PCR using internal gene-specific and vector-specific primers (PCR2). The region encompassing the initiation codon was obtained by sequencing a cosmid subclone (CosI1Xho4;2; Fig. 1).

**Organization of RTK6 Gene Product.** The complete nucleotide sequence (nucleotides 1–3841) has one long open reading frame (nucleotides 337–2964) that is followed by a poly(A)* signal (nucleotide 3807) and a polyadenine tract. The first in-frame methionine (at nucleotide 337) has the Kozak consensus sequence and is preceded by several stop codons (20). The 5' untranslated sequence has a highly GC-rich region characteristic of several other genes. The 3' untranslated region extends over 1 kb from the in-frame stop codon to the poly(A)* tail. (The complete sequence has not been shown but has been submitted to the EMBL database with accession number Z29093).

The predicted amino acid sequence of RTK6 has all of the features suggestive of a typical transmembrane receptor, i.e., a signal peptide is followed by extracellular, transmembrane, and cytoplasmic domains. The unprocessed RTK6 would be a protein of 876 amino acids with a predicted molecular weight of 97,000 daltons. The catalytic domain is 49% identical with the TRK-2h protein catalytic domain, but the remainder of the protein is dissimilar (21). The COOH-terminal tail following the catalytic domain is short (8 amino acids) and similar to the TRK-2h protein (Fig. 2). There are several possible autophosphorylation sites within the catalytic domain (tyrosine residues 755, 759, and 760) which are also conserved in the insulin receptor (Ref. 22; Fig. 2). The NHR-terminal region has an unusual domain (residue 31–185) which has strong homology to a repeating domain found in factor VIII, Factor V, milk fat globule membrane 2 protein, and the A5 antigen (Fig. 3). This domain is repeated twice in all of these proteins and is separated by a short stretch of amino acids. It is also present in the protein discoidin I, found in Dictyostelium discoideum. The alignment of sequences show that RTK6 is similar in all respects to the recently described receptor kinase DDR, except for a short stretch of 37 amino acids which is absent (between nucleotides 1653–1764 of the DDR sequence) in RTK6 (Ref. 23; Fig. 4a). This region is in the juxtamembrane region of the protein. There are two other amino acid differences between RTK6 and DDR (residue 94 leucine instead of valine; residue 796 leucine instead of valine). The difference in cDNA between DDR and RTK6 in the juxtamembrane region (nucleotides 1653–1764 of the DDR sequence; Fig. 4a) was investigated further by isolation of genomic sequence around this region. A cosmid subclone encompassing this region was obtained from a PstI digest of Cos1. Sequence analysis showed that the 37 amino acids present in DDR and absent in RTK6 were coded by one exon. It was possible to determine the intron-exon boundary around this exon by comparison with the cDNA sequence (Fig. 4b). Thus, the difference at the cDNA level between RTK6 and DDR can be explained by alternative splicing of this exon.

Another recently described kinase, TKT, has strong homology (73% amino acid) to both RTK6 and DDR, suggesting it is another member of the same subfamily (Ref. 5; Figs. 2 and 3).

**Expression.** In a panel of human tissues, Northern analysis of RTK6 shows a single transcript of 4.5 kb, present in low levels in heart, placenta, lung, liver, muscle, kidney, and pancreas, with high levels of expression in the brain (Fig. 5). The ribonuclease protection assay (using a short fragment from the 3' untranslated region as an antisense riboprobe) using total cellular RNA from a panel of ovarian cancer cell lines shows variable expression of RTK6 compared to the GAPDH RNA in each sample. Two breast cell lines also showed expression of the RTK6 message (Fig. 6). There was a two- to three-fold variation in mRNA levels compared to placenta as evaluated by densitometry.

To determine the expression of the exon in the juxtamembrane region of DDR, which was absent in RTK6, RT-PCR was performed with total cellular RNA from a panel of ovarian cell lines with flanking oligonucleotide primers around the exon. The predicted size of the amplified product for RTK6 was 496 bp and for DDR was 607 bp. Both products were amplified in all of the ovarian cell lines and placenta. (Fig. 7) and confirmed by hybridization to contain their respective sequences.

**In Situ Hybridization.** In order to assess the localization of RTK6, in situ hybridization was performed in a number of tissues. The results show expression in the epithelial cells of the ovary, fetal lung, small bowel, thymus, fetal kidney, and in the gray matter of fetal brain (Fig. 8, a–e). A panel of ovarian tumors (two benign, two borderline, and two malignant) were examined for expression of RTK6 message. There was low to absent signal in normal, benign, and borderline tumors (Fig. 8, f–h). However, in a well-differentiated malignant tumor, there were areas of comparatively high signal strength in the malignant cells (Fig. 8i). To evaluate the expression of RTK6 further, a panel of 27 epithelial ovarian tumors of different grades (2 well-differentiated, 7 moderately differentiated, 13 poorly differentiated malignant, and 5 borderline epithelial ovarian tumors) and histological type were examined by in situ hybridization. There was no clear pattern of expression correlating with histological grade, although maximal expression relative to β-actin message was observed only in moderate and poorly differentiated malignant tumors (Table 1).

**Biochemical Characterization.** To identify the protein encoded by RTK6, polyclonal antisera (6.1 and 6.2) were prepared in rabbits using thyroglobulin-conjugated synthetic peptides (residues 478–495 and 848–863, respectively) from the juxtamembrane and COOH-terminal re-

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4. S. Laval, R. Butler, and T. S. Ganesan, unpublished observations.
**Fig. 2.** Alignment of sequences within the catalytic domain between RTK6, DDR, TKT, insulin receptor (Ins) (22), insulin receptor-related receptor (Int) (50), trk-2h (21), and c-met (Met) (51). Identical and conserved residues are shown as boxed in black and gray, respectively. The alignment was performed using the GCC program Pileup and Lineup (49).

**Fig. 3.** The homology (amino acid level) between RTK6, DDR, TKT, factor V, factor VIII, milk fat globule membrane protein, A5 antigen, and discoidin I. The sequences from the EMBL database were aligned using GCC program Pileup and Lineup (49). Regions of identical or conserved residues are boxed in black and gray, respectively. The two repeat domains in factor V, factor VIII, MFGA, and A5 are shown as 1 and 2.
A

**Fig. 4.** A, diagram showing the absence of 37 amino acids in RTK6 compared to the published DDR sequence (23). The bottom panel illustrates the exact position of the divergence from RTK6 within the DDR nucleotide sequence. B, sequence of the genomic region around the divergence between RTK6 and DDR. The exon sequence is shown in upper case and the intron sequence in lower case letters. The amino acid sequence is shown in single-letter code, below the exon under the first base of the codon. The exon corresponding to the 37 amino acids in DDR is underlined.

Discussion

There are several classes of receptor tyrosine kinases based on the sequence motifs within the catalytic domain and the extracellular domain. The ability to isolate new members by PCR using degenerate oligonucleotide primers based on the conserved motifs within the catalytic domain has been successful in illustrating the diversity of tyrosine kinases. There are now at least 10 classes of receptors based on the differences within the extracellular domain (5). These structural motifs vary between immunoglobulin-like domains, fibronectin domains, and cysteine-rich domains. The different motifs probably associate with different ligands in different contexts of cellular metabolism, thus reflecting their participation in a wide variety of cellular functions. The receptor tyrosine kinase described in this study belongs to a novel subfamily which is distinguished by a novel extracellular domain. The extracellular region is composed of a domain which is observed as tandem repeats in the COOH-terminal region of several apparently unrelated proteins. These are factor VIII, factor V, milk fat globule membrane
proteins, and A5 antigen (14, 16, 17, 18, 24). The functional role of these domains is not understood completely; however, they are homologous to the lectin Discoidin I found in D. discoideum which participates in cell aggregation (13). It is thought that this domain in D. discoideum is important in cell-cell contact, promotes cellular migration, and has similar functions to fibronectin. This domain has also been shown to be important in the interaction of factor VIII with phospholipid and may have a similar role in the milk fat globule membrane proteins (17, 25, 26). All of the proteins which share a homology with RTK6 in this domain are relatively abundant in epithelial cells, which suggests that RTK6 may also serve as a specific molecule which functions in cell-cell contact signaling pathways.

The juxtamembrane region is unusually proline and glycine rich, as well as exceptionally long for any receptor tyrosine kinase described thus far. Such a highly proline- and glycine-rich region has not been described for an integral membrane protein. By analogy to adaptins which have such regions, this might serve to anchor other transmembrane proteins to clathrin-coated pits and vesicles (27, 28). RTK6, which has an otherwise similar sequence with DDR, differs in the juxtamembrane domain (Fig. 4, a and b; Ref. 23). The absence of 37 amino acids in RTK6 in the juxtamembrane domain compared to DDR involves the NPXY motif, which has been suggested as an important requirement for internalization in coated pits (29, 30). This motif has been observed in other receptor tyrosine kinases and the low density lipoprotein receptor. However, in receptor tyrosine kinases, unlike the low density lipoprotein receptor, it may not be important for ligand-dependent events. Analysis of the juxtamembrane domain has been performed by site-directed mutagenesis to understand the role of phosphorylation of specific tyrosine residues in ligand-dependent internalization of the receptor. In the insulin receptor, the NPXY motif alone is not responsible for internalization, nor are phosphorylation of individual tyrosine residues within the juxta-membrane domain (31). However, it has been demonstrated in the platelet-derived growth factor-β receptor that phosphorylation of tyrosine residue 579 is important for internalization (32). Because this region was the major difference between RTK6 and DDR, further analysis was undertaken at the genomic level.

The isolation of genomic sequences confirms that a single exon is probably alternatively spliced to give rise to RTK6 (Fig. 4b). The 5’ splice site shows good agreement with the consensus sequence (exon-AG/guaagu-intron). The 3’ splice site differs from the consensus site at the first base of the exon (intron-(Y), ag/G-exon; Ref. 33).

The single mRNA species detected by Northern analysis is similar to that reported for DDR. The relatively abundant message in the brain suggests that, like TRK, it may have an important role in neuronal differentiation (Ref. 34; Fig. 5). In a panel of ovarian cell lines, there was varying expression of RTK6 compared to placenta. As reported, two breast cell lines also showed expression of RTK6 (Fig. 6). Further analysis of the difference between RTK6 and DDR was undertaken by examining their expression in a panel of cell lines using RT-PCR and oligonucleotide primers flanking the putative alternatively spliced exon (Fig. 4b). The sensitive RT-PCR analysis of a panel of ovarian cell lines demonstrates that the two forms are expressed in all cells (Fig. 7). It has to be confirmed by ribonuclease protection assay whether both forms are equally expressed in different tissues or if the expression of the exon is tissue specific. It will be important to compare the relative biological roles of RTK6 and DDR, particularly with respect to ligand-dependent internalization of receptor.

The demonstration of RTK6 message by in situ hybridization clearly localizes it to epithelial cells (Fig. 8). The message is particularly abundant in lactating mammary gland and in intestinal crypts. The similarity with the milk fat globule membrane proteins in the discoidin domain and their presence in the lactating breast might be more than just a coincidence. In the ovary, the expression of RTK6 message, which is weak in the epithelial cells of benign and borderline tumors, is comparatively increased in the malignant tumor. A larger panel of malignant and borderline tumors of the ovary confirmed this preliminary observation. However, in individual malignant tumors of different histological type and grade, no correlation could be observed with the expression of RTK6 (Table 1).

The protein detected by the 6.2 antibody (M, 140,000; Fig. 9) in 41M cells is higher than the predicted molecular weight of the protein. This might reflect additional post-translational changes in vivo in cells. There are at least four potential glycosylation sites within the extracellular domain, and it is possible that this accounts for the increase from the predicted molecular weight of the protein. The intrinsic tyrosine kinase activity of the protein encoded by RTK6 is confirmed by the ability to autophosphorylate itself (Fig. 10). However, this activity is weak and may be difficult to demonstrate in cancer cells in the absence of a cognate ligand. This, however, can be analyzed further by transfection of the cDNA into appropriate eukaryotic cell lines. A recent report showed the absence of receptor (p140trkA) for the NGF in normal human keratinocytes where NGF is expressed and binds to high and low affinity receptors (35). They isolated a new member of the trk family, which is expressed in keratinocytes and suggested that it may be another receptor for NGF. In searching the

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1 A. M. Hanby, R. Poulsom, and T. S. Ganesan, Expression of EDDR1 in normal and malignant breast disease, manuscript in preparation.
GenBank and EMBL databases, RTK6 is identical in sequence to this new gene trkE (36). Thus, it is possible that the NGF has other functions in epithelial cells, and this may be mediated by RTK6/trkE. This could now be experimentally tested with stimulation of cells by NGF.

Another member of this family, TKT, was reported recently which shares 73% homology with RTK6 (Figs. 2 and 3), which also has a factor VIII-like domain (5). Thus, there are at least two members of this subfamily. Further experiments to characterize the ligand of this molecule and the downstream events after stimulation would reveal details about the function of this protein and how it is altered in malignancy. Due to the simultaneous isolation of this phosphoprotein by several groups (at least two in the database) in different cells and contexts, it is suggested that RTK6 be named according to its apparent location and the unusual NH2-terminal discoidin-like domain, epithelial discoidin domain receptor, and the family which should include TKT be called discoidin domain receptors.

Materials and Methods
Isolation of cDNA Clones and Sequencing. The two degenerate oligonucleotide primers (PTK I and II) used in the PCR were designed based on the two consensus motifs within the catalytic domain of all protein kinases, IHRDL and DVWSFG (12). PCR was performed using a cDNA library of SKOV-3 (in pKS vector, a derivative of CDM8; 50 ng), with deoxynucleotide triphosphates (200 μm), primers (1 μg each), MgCl2 (1.5 mM), KCl (25 mM), Tris-Cl (pH 8.3, 20 mM), and Taq polymerase 2.5 units (Cetus). The PCR cycle was 45 s at 94°C (denaturation), 2 min at 37°C (annealing), and 3 min at 63°C (elongation) for 30 cycles, and a final extension step at 72°C for 10 min. The PCR product was purified using a Sephadex G-50 column, the DNA ethanol was precipitated. The purified DNA was ligated to EcoRV-digested pBluescript SK (Stratagene) and transformed into competent DH5α Escherichia coli cells according to manufacturer’s instructions. White colonies were picked and amplified by PCR using M13 forward and reverse sequencing primers. Fifty clones with inserts above 210 bp (the predicted size between the conserved motifs) were analyzed further. PCR of individual clones was performed using biotinylated M13 FSP, purified using Dynabeads, and sequenced using the T7 primer. The sequence was compared in each individual clone with known protein kinases in the EMBL and GCG database using the MacVector program. Two novel RTKs (RTK6 and RTK15) were identified, and RTK6 was selected for further characterization.

Using clone 6DIF as a probe, the cDNA library of SKOV-3 was screened by hybridization as described (37). Two positive overlapping clones (6D and 6DIF) of 1 and 1.8 kb, respectively, were identified. An additional 1 kb 5′ clone was obtained from a placental cDNA library by PCR using nested primers from within clone 6D and the vector
Fig. 8. *In situ* hybridization of a panel of human tissues and ovarian tumors using a $^{35}$S-labeled antisense riboprobe (clone 6D). Each pair of photographs shows both light and dark fields. a, fetal kidney; b, fetal brain; c, small bowel; d, fetal lung; e, thymus; f, a simple ovarian cyst; g, benign cystadenoma of the ovary; h, borderline tumor of the ovary; i, well-differentiated serous adenocarcinoma of the ovary. The dark field photographs show localization of message to the epithelial cells as bright spots. In b, the arrow points to the message within brain. Magnification before photography was between $\times$ 10 and $\times$ 20.
Table 1 Expression of RTK6 as determined by in situ hybridization on a panel of ovarian tumors

<table>
<thead>
<tr>
<th>Name</th>
<th>Histological type</th>
<th>Grade</th>
<th>Expression*</th>
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<tr>
<td>S. R.</td>
<td>Mucinous adenocarcinoma</td>
<td>–</td>
<td>+/-</td>
</tr>
<tr>
<td>U. K.</td>
<td>Mucinous adenocarcinoma</td>
<td>–</td>
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<tr>
<td>J. T.</td>
<td>Serous adenocarcinoma</td>
<td>–</td>
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<tr>
<td>J. G.</td>
<td>Mucinous adenocarcinoma</td>
<td>–</td>
<td>–</td>
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<tr>
<td>B. A.</td>
<td>Mucinous adenocarcinoma</td>
<td>–</td>
<td>+</td>
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<td>Endometroid adenocarcinoma</td>
<td>Well-differentiated</td>
<td>++</td>
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<tr>
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<td>–</td>
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<td>S. B.</td>
<td>Mucinous adenocarcinoma</td>
<td>Moderate diff.</td>
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<td>Endometroid adenocarcinoma</td>
<td>Moderate/Focally</td>
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<td>+++</td>
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<td>+++</td>
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<td>P. S.</td>
<td>Serous adenocarcinoma</td>
<td>Poorly diff.</td>
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*+*, presence of message; **-,** absence of message. Relative strength of message was always confirmed by hybridization with β-actin probe.

**b** Refers to borderline histology in all of the five tumors.

diff., differentiated.

primer. After several cDNA libraries were screened for further 5' sequences without success, three overlapping cosmids clones were isolated from a human chromosome 6 cosmid library to which the gene maps (38, 39). Cos1 was digested with XhoI and ligated into pBluescript SK. A cosmid subclone Cos1Xho 4.2 was sequenced using gene-specific primers to identify the remaining 5' sequence and to identify the initiation codon. The genomic sequences corresponding to the difference between DDR and RTK6 were isolated from a PstI subclone of Cos1.

Sequencing was performed on both strands using the Sequenase kit (USB) and the dyeoxy chain termination method (40). The cDNA was subcloned for sequencing into pBluescript SK and was sequenced using both oligonucleotide primers and nested deletions. All oligonucleotide primers were synthesized on an Applied Biosystems 380A synthesizer using standard chemistry.

**Northern Analysis.** A human multiple tissue Northern blot (Clontech labs) containing 2 µg of poly(A)^+ RNA from eight different tissues was used. Hybridization was performed with the original RTK6 PCR (400-bp) fragment as described (35). The probe was labeled using the random prime labeling technique with [α-32P]dCTP (Amersham). After overnight hybridization at 42°C, the filter was washed stringently with a final wash at 65°C using 0.1X standard saline citrate/0.1% SDS before exposure to Kodak X-ray film with two intensifying screens at ~70°C.

**Ribonuclease Protection Assay.** A 254-bp RsaI fragment (nucleotides 3465–3719) from clone 6DIF was subcloned into pBluescript SK. The plasmid was linearized with EcoRI, and the assay was performed as described in the manufacturer's protocol (USB lysate ribonuclease protection kit) on total cellular RNA from a panel of eight ovarian cancer cell lines, two breast cell lines, and placenta. GAPDH probe was used to control for RNA loading and integrity.

**RT-PCR.** Total cellular RNA was isolated from a panel of ovarian cell lines by standard techniques. The first-strand cDNA synthesis was performed using random hexamers, deoxynucleotide triphosphates, Superscript RT (GIBCO-BRL) and buffers according to the manufacturer’s instructions with 1 µg of total RNA in a volume of 20 µl. PCR was performed in a total volume of 50 µl using primer A (5'-CAGATCATCCATCAACAACCG-3') and primer B (5'-TCAGGAATACATTGCTGGC-3') corresponding to nucleotides 1740 and 2236, respectively, of the cDNA sequence of RTK6. The reaction conditions for 35 cycles were denaturation at 94°C for 45 s, annealing at 60°C for 60 s, and extension at 72°C for 2 min, with a final extension step for 10 min.

**In Situ Hybridization.** In situ hybridization was performed as described previously (41). The antisense probes were labeled with [32P]UTP (800 Ci/mmol; Amersham). Briefly, 4 µm sections were cut from formalin-fixed, paraffin-embedded blocks on TESPA (3-amino-propyl triethoxysilane; Sigma)-treated slides and dried overnight at 37°C. Sections were dewaxed in xylene and then hydrated in decreasing concentrations of ethanol. Tissues were permeabilized with proteinase-K, fixed in 4% paraformaldehyde, acetylated, and air-dried before being hybridized overnight with 1–2 x 10^6 cpm of probe in 10–20 µl of hybridization mix at 55°C. The slides were washed extensively with 50% formamide/4X SSC at 55°C, followed by an RNase step;
washing was continued, reaching a final stringency of 0.5X SSC at 65ºC. Slides were dipped in Ilford K5 emulsion, and autoradiography was at 4ºC for approximately 4 weeks. Counterstaining was performed with dilute Giemsa. Sections were examined in dark field conditions using an Olympus BH2 microscope. As a control for the presence of RNA in all tissue compartments, sections from all blocks were hybridized with a β-actin probe.

The template for RTK6 was EcoRV linearized clone 6D in pBluescript SK. The transcript was made with T7 RNA polymerase and contained 84 bases of CDM8 and Bluescript vector sequences in addition to sequences of RTK6. The template for the β-actin probe using SP6 RNA polymerase was phBA-10 linearized with DraI, which was prepared by subcloning the 450-bp EcoRI/Rsal fragment of human β-actin sequence present in pATh 3’UT (42) into the EcoRI/EcoRV sites of pSP73 (Promega).

**Polyclonal Antiserum.** Peptides corresponding to residues 478–495 and 848–863 (6.1 and 6.2) were chosen from the predicted amino acid sequence of RTK6, based on hydrophilicity and antigenicity using the GCG program Peptide Structure. Peptides were synthesized and conjugated to thyroglobulin for immunization of rabbits as described previously (43). Polyclonal antisera were checked for specificity and titer by enzyme-linked immunosorbent assay against peptide, and the antibodies were purified by affinity chromatography using the Immunopure Ag/Ab immobilization kit according to manufacturer’s instructions (Pierce).

**Protein Kinase Assay.** 41M cells (5 x 10⁶) were solubilized in lysis buffer [1% Triton X-100, 10 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, 250 mM phenylmethylsulfonyl chloride, 70 µg/ml aprotinin, 100 µM sodium vanadate, and 1 mM okadaic acid] for 30 min at 4ºC. Lysates were centrifuged for 30 min at 10,000 rpm at 4ºC. The supernatant was incubated with 10 µg of purified antibody or 10 µg of preimmune serum with 50 µl of protein A-Sepharose beads for 1 h at 4ºC. In vitro phosphorylation of washed precipitates were performed with 20 mM each of MnCl₂ and 1,4-piperazinediethanesulfonylic acid using 10 µCi of [γ-32P]ATP (Amersham) at 30ºC for 10 min. After washing twice with lysis buffer, the beads were boiled in 50 µl of 2X SDS/PAGE sample buffer. SDS-PAGE (6%) was performed as described (44).

**35S Metabolic Labeling of Cells.** One plate of 41M cells (5 x 10⁶) was washed twice in methionine-free Dulbecco’s modified Eagle’s medium with 5% fetal calf serum and incubated for 30 min. The cells were labeled in 4 ml of methionine-free medium with 5% fetal calf serum with 1 mCi of [35S]methionine (1055 Ci/mmol; ICN) for 3 h. The cells were washed twice in ice-cold phosphate-buffered saline and solubilized in lysis buffer at 4ºC for 1 h. The lysates were centrifuged at 10,000 rpm for 30 min. The supernatant was precleared with protein A-Sepharose beads overnight and with preimmune serum for 2 h. Immunoprecipitation was performed with 10 µl of preimmune serum or 10 µg of purified 6.2 antibody for 3 h with 50 µl of protein A-Sepharose beads. The beads were washed in lysis buffer, Tris saline, and 0.05 M Tris (pH 6.8), respectively. Reduced samples were run on 6% SDS-PAGE, and the gel was incubated with Entensi (Dupont) before fluorography at −70ºC using intensifying screens and Kodak X-ray films.

**Cell Lines.** Human cell lines were grown at 37ºC in a humidified atmosphere of 5% carbon dioxide and maintained in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum with the following supplements: 10 mM glutamine, 100 units/ml penicillin, and 12.5 µg/ml streptomycin. The ovarian cancer cell lines SKOV-3, PeO1, PeO4, PeO14, 41M, 59M, OAW28, and OAW42 have been described previously (19, 45, 46). The breast cell lines used were ZR75-1 and MDA-231 (47, 48).

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