H-RYK, an Unusual Receptor Kinase: Isolation and Analysis of Expression in Ovarian Cancer

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ABSTRACT

Background: 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. Receptor tyrosine kinases (EGF receptor and c-erbB2) have been shown to be important in the pathogenesis of cancer. In ovarian cancer, overexpression of c-erbB2, a type I receptor, has been correlated with an adverse effect on survival of patients.

Material and Methods: An unusual receptor tyrosine kinase, H-RYK, has been isolated from a complimentary DNA library of SKOV-3, an epithelial ovarian cancer cell line, using a polymerase chain reaction–mediated approach.

Results: The primary structure of the predicted amino acid sequence of the protein shows a novel NH_2-terminal region. The catalytic region shows homology to other tyrosine kinases, the closest homology being with v-sea (39%). A significant alteration in the catalytic domain is that the highly conserved "DFG" triplet in subdomain VII is altered to "DNA." The gene was mapped to chromosome 3q22. A single transcript of 3.0 kb is expressed in heart, brain, lung, placenta, liver, muscle, kidney, and pancreas by Northern analysis with maximal expression in skeletal muscle. In situ hybridization analysis on human tissues demonstrated localization of message in the epithelial and stromal compartment of tissues such as brain, lung, colon, kidney, and breast. There was minimal to absent expression of H-RYK on surface epithelium of ovaries. In benign (3) and borderline tumors of the ovary (5), there was expression in the stromal compartment. However, in malignant tumors (24) there was increased expression predominantly confined to the epithelium. Polyclonal antisera raised against synthetic peptides recognize a 100-kD protein in ovarian cancer cells and other cell lines. In contrast to other receptor tyrosine kinases, the receptor did not phosphorylate in an in vitro kinase assay.

Conclusions: The expression of this unusual receptor tyrosine kinase in epithelial ovarian cancer suggests that it may be involved in tumor progression, which needs further investigation.

INTRODUCTION

Protein tyrosine kinases (PTK) represent a large family of enzymes that share a common highly conserved catalytic domain responsible for signal transduction by phosphorylation of substrates on tyrosine residues (1,2). Receptor tyrosine kinases (RTK) are transmembrane proteins that relay the signals from the exterior of the cell, upon stimulation by cognate ligands. These signals are instructions to the cell for various fundamentally important processes such as division and differentiation. The extracellular domain determines

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the specificity of the ligand they bind to, which can be used to subdivide them into different families (3). The downstream signaling pathways have been defined to occur through association with *src* homology 2 (SH$_2$) domain containing proteins, via *ras* and the MAP kinases, and also through the STAT kinase pathway, to ultimately stimulate transcription (4–7). Deregulation in any component of this pathway at the level of receptor or downstream elements has been shown to be important in malignant transformation (8). At the level of receptors for example, overexpression of RTKs of the type I class, like EGF and c-*erb*B2, have been implicated in the pathogenesis of human breast and ovarian cancer (9). Insulin-like growth factor-1 (IGF-1) receptor expression has been shown to be essential for maintenance of the transformed phenotype (10). Recently, "ret", another class of RTK, has been shown to be important in the pathogenesis of multiple endocrine neoplasia due to mutations in the catalytic domain which alter substrate specificity (11–13). Mutations in the fibroblast growth factor receptor family also cause human disease, such as achondroplasia (14). Thus, RTKs have been shown to be important in the pathogenesis of cancer and other diseases.

Recently, several approaches have been employed to isolate novel receptor tyrosine kinases. The most successful approach has been that based on using the polymerase chain reaction (PCR) and degenerate oligonucleotide primers corresponding to the conserved amino acid motifs within the kinase domain (2,15). This has led to the isolation of several new tyrosine kinases of receptor and nonreceptor types. Adopting a similar strategy, two RTKs, EDDR1 (16) and H-RYK, were identified from an epithelial ovarian cancer cell line, SKOV-3. This paper reports the isolation, expression, and biochemical characterization of H-RYK. During the course of this investigation, an identical human cDNA was published, and hence we have chosen to use the same name, H-RYK (17,18). This represents a new class of receptor tyrosine kinases, with unusual sequence alterations in conserved motifs within the catalytic domain. The expression of H-RYK is localized to epithelial and stromal cells and is increased in malignant ovarian tumors. It codes for a 100-kD protein, which is expressed ubiquitously, but does not autophosphorylate in an in vitro kinase assay.

MATERIALS AND METHODS

Isolation of cDNA Clones and Sequencing

RTK 15 was isolated from the SKOV-3 cDNA library as previously described (16). All isolated cDNA clones were fully sequenced in both strands using a Sequenase kit (USB, Cleveland, OH, U.S.A.) and the dideoxy chain termination method (19). Sequencing was performed using both oligonucleotide primers and M13 FSP/RSP primers on pBluescript SK. All oligonucleotide primers were synthesized on an Applied Biosystems 380A synthesizer using standard chemistry.

Northern Analysis

A human multiple tissue Northern blot (Clontech Labs, Palo Alto, CA, U.S.A.) containing 2 µg of poly(A)$^+$RNA from eight different tissues was used. Hybridization was performed with the original RTK15 fragment as a probe and confirmed subsequently with the full-length cDNA. The probe was labeled using the random prime labeling technique with [α^{-32}] dCTP (Amersham, U.K.), and hybridization conditions were as described (16).

In Situ Hybridization

In situ hybridization was performed as described previously with some modifications (16,20). The template for RTK15 was *Eco*RV linearized clone 15.5BX3 in pBluescript SK. The transcript was made with T$_7$ polymerase. As a control for the presence of RNA in all tissue compartments, sections from all blocks were hybridized with a β-actin probe (16).

Polyclonal Antisera

Peptides corresponding to residues 321–337 and 551–568 (15.1 and 15.2) were chosen from the predicted amino acid sequence of H-RYK, 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 (21). Polyclonal antisera were checked for specificity and titer by enzyme-linked immunosorbent assay against peptide, and the antibodies were purified using the Immunopure Ag/Ab immobilization kit according to manufacturer's instructions (Pierce, U.K.).

Western Blotting

41M cells (5 × 10^7) were solubilized in lysis buffer (containing 1% Triton X-100, 10 mM sodium phosphate buffer [pH 7.0], 150 mM Nacl, 250 mM phenylmethylsulfonyl chloride, 70 μg/ml aprotonin, 100 μM sodium vanadate, 1 μM okadaic acid, 100 μM bis [p-nitro-phenyl] phosphate, 2.5 μg/ml leupeptin, 2.5 μg/ml trypsin inhibitor, and 2.5 μg/ml pepstatin A) for 30 min at 4°C. Lysates were centrifuged for 30 min at 13,000 rpm at 4°C. The supernatant was incubated with 1 μg of purified antibody with or without 100 μg of cognate peptide. Peptide and antibody were usually preincubated for 1 hr at 4°C before adding to supernatant and protein-A sepharose beads for 2 hr at 4°C. Beads were centrifuged and washed twice with lysis buffer before being boiled 5 min in 40 μl of 2× SDS/PAGE sample buffer. SDS/PAGE (6%) was performed as described (22).

Western blotting was performed using Amersham's ECL hybond filter overnight (12–18 hr) at 15 V in Tris-Glycine buffer. The filter was incubated with primary and secondary antibodies using the enhanced chemiluminescence kit according to manufacturer's instructions (Amersham). The primary antibody (15.1/15.2) was used at a concentration of 1 μg/ml and the secondary antibody (anti-rabbit polyclonal) at 1:5000 dilution. Autoradiographs were exposed from 1 min up to 1 hr for optimal signal.

Transfection

The entire cDNA (15SC) was cloned into plasmid (pcDNA1) under the control of a CMV promoter. Chinese hamster ovary cells were grown to subconfluence in 90-mm dishes using Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The medium was aspirated from the dishes and 4.75 ml of DMEM was added together with the transfection cocktail (5–20 μg of cDNA in 5 μl of sterile distilled water, 40 μl phosphate-buffered saline (PBS), 200 μl DEAE Dextran (10 mg/ml), 5 μl chloroquine) and mixed by swirling. The cells were incubated for 2 hr; followed by washing once with PBS. Five milliliters of PBS plus 10% DMSO was added and the cells were incubated at room temperature for 1 min. Subsequently, cells were washed twice with PBS and then cultured again in DMEM with 10% FCS at 37°C for 48 hr, before harvesting them with trypsin and EDTA for assaying for protein expression by Western blotting. Processing of the cells was performed as described above.

Chromosomal Localization

Metaphase spreads were examined from PHA-stimulated normal human lymphocytes using standard cytogenetic techniques (23). The entire clone 15.5BX3 was used as a probe for fluorescent in situ hybridization (FISH) as described (24).

Cell Lines

Human cell lines were grown at 37°C in a humidified atmosphere of 5% carbon dioxide and maintained in DMEM and 10% FCS 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, OAW42, and UC101 have been described previously (16). K562, HSB2, U937, Daudi, HL60, HeLa, C6, 293, BRL, and NIH3T3 were obtained from Cell Production Services at ICRF.

RESULTS

Isolation of H-RYK cDNA Clones

A short PCR fragment of 210 bp (RTK 15) was identified from a SKOV-3 cDNA library, using techniques described previously (16). Sequencing of this fragment showed a novel sequence unrelated to previous kinases. This was used as a probe, and two clones 15SC (2.3 kb) and 15P1 (2.1 kb) were identified. Sequencing of 15SC showed that it contained a continuous open reading frame. There was variation in the 3′ region due to the presence of longer untranslated sequences in 15P1. The clones were identical in sequence in the coding region except for an in-frame 9-bp insertion in 15.5BX3 between nucleotides 868 and 876 resulting in the addition of three amino acids, ser-leu-gly. Comparison of our sequence with that of the earlier published sequence (17,18) showed that they were identical except for some individual amino acid differences. The entire nucleotide and the putative amino acid sequence have been deposited with the EMBL database.
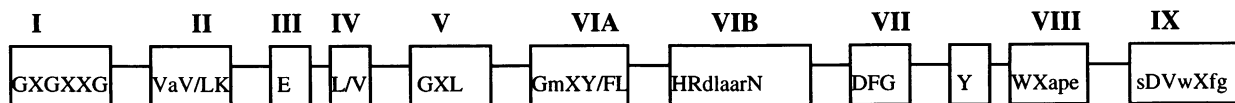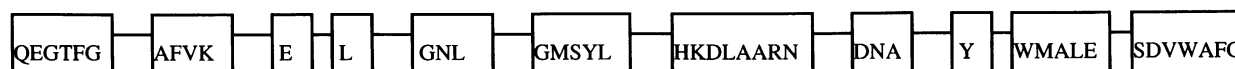
a. Conserved Protein Kinase Domains (I-IX)

I	II	III	IV	V	VIA	VIB	VII	VIII	IX
GXGXXG	VaV/LK	E	L/V	GXL	GmXY/FL	HRdlaarN	DFG	Y	WXape	sDVwXfg

b. H-RYK Protein Kinase Domains (I-IX)

QEGTFG	AFVK	E	L	GNL	GMSYL	HKDLAARN	DNA	Y	WMALE	SDVWAFG

FIG. 1.   Comparison of the catalytic domain of H-RYK with the conserved amino acids in the protein kinase family (2)

Organization of H-RYK Protein

The predicted amino acid sequence of the putative H-RYK protein has all the features suggestive of a transmembrane receptor. This includes an extracellular domain, transmembrane domain and a conserved catalytic domain. The N-terminal domain is novel and short compared with other kinases and has at least five glycosylation sites. There is also a stretch of four basic amino acid residues KRRK (amino acid 159–162) which probably represents a potential proteolytic cleavage site as found in the insulin receptor (25). The catalytic domain is about 39% identical to the v-*sea* oncogene, but the rest of the protein is dissimilar (26). The carboxy-terminal region is only 19 amino acids long and short compared with other kinases. The critical changes in the catalytic domain compared with other kinases are shown in Fig. 1.

There are several tyrosine residues which are potential phosphorylation sites within the juxta-membrane and the catalytic domain. It is now possible to predict possible sites for binding to SH$_2$ domain containing proteins based on the three amino acid sequences carboxy-terminal to the tyrosine (13,27,28). There were four such binding sites identified within the catalytic domain which had good consensus sequences to bind to Grb-2, SHPTP2, Syk, and Vav SH$_2$ proteins. The corresponding motifs within H-RYK are YMNW (tyrosine 386); YVDI and YHCL (tyrosine 516 and 468); YHCL (tyrosine 468); and YMNW (tyrosine 386) respectively for each SH$_2$ domain protein. Such motifs are usually found outside the catalytic domain in other RTKs.

Chromosomal Localization of H-RYK

The chromosomal localization of the H-RYK was determined by FISH analysis on normal human metaphase spreads using 15.5BX3 as a probe and was shown to map to 3q22 (Fig. 2). In contrast to the earlier report (17), no signals were identified on chromosome 17.

Expression of H-RYK

The clone 15SC was hybridized to poly(A)$^+$RNA from a panel of normal tissues and detected a single 3.0-kb transcript (Fig. 3). The gene was expressed in all tissues, but particularly strongly in skeletal muscle. To evaluate the significance of the 9-bp difference in the juxta-membrane domain between cDNA clones of H-RYK, reverse transcriptase PCR was performing using flanking primers. The two expected fragments were demonstrated in equal amounts from RNA of a panel of ovarian cell lines, and cDNA libraries (data not shown). The expression of H-RYK was also investigated by ribonuclease protection assay using a fragment from the 3' untranslated region of 15.5BX3 as an antisense probe in a panel of eight ovarian cell lines. There was equivalent expression of message in
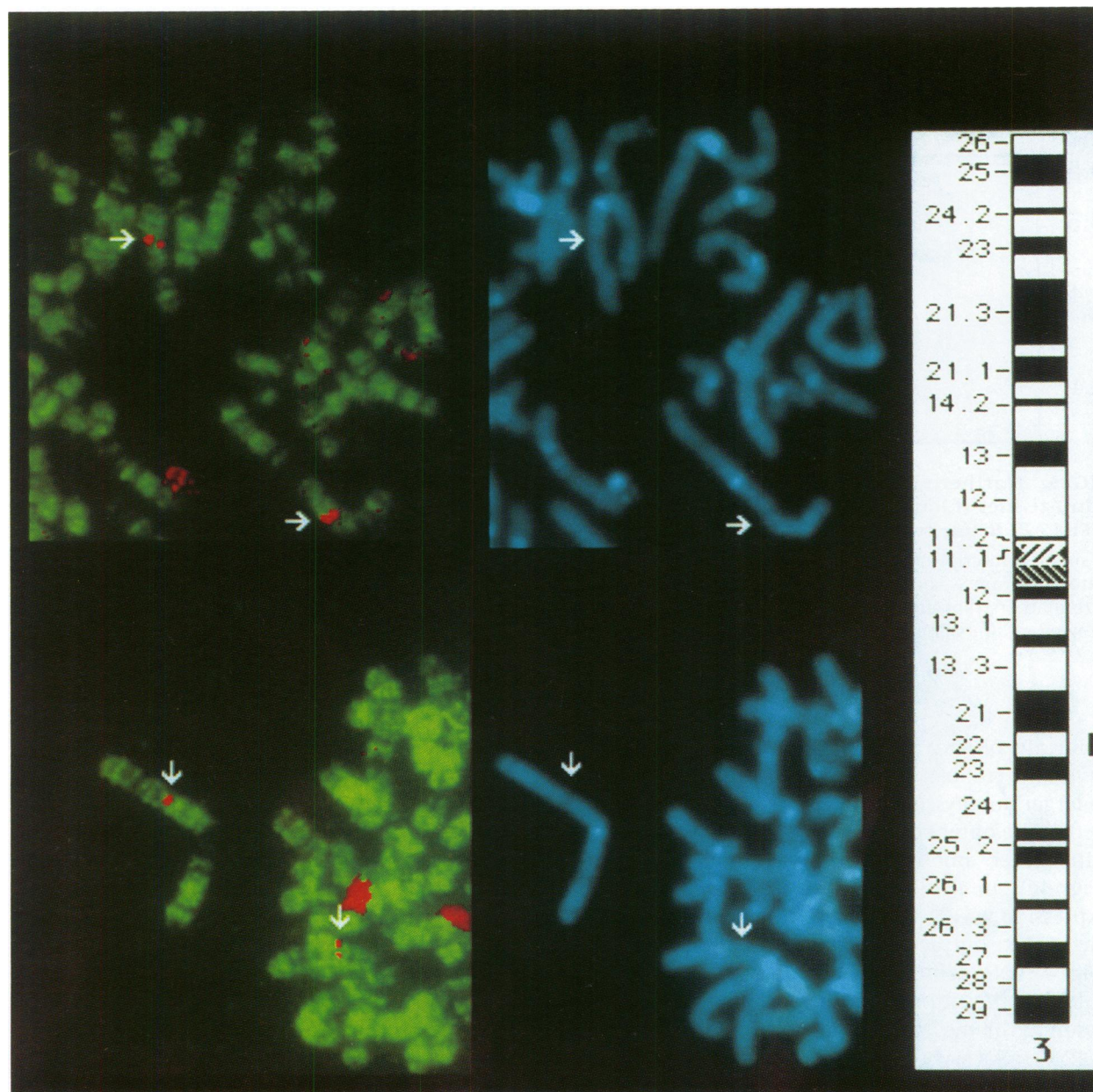
FIG. 2. Two partial metaphase spreads showing localization of probe to human chromosome 3q24 (arrowed)

Signal was detected with Texas red on R-banded chromosomes stained with anti-bromodeoxyuridine-FITC. DAPI counterstaining of the same spreads produced a G-banding pattern. Metaphase spreads were processed using a Zeiss Axioscop microscope equipped with a CCD camera (Photometrics, Photometrics, AZ, U.S.A.). Separate images of probe signal, banding pattern, and counterstain were captured and colored. These images were then merged using a computer software developed by T. Rand and D. C. Ward (Yale University, New Haven, CT).

all the cell lines compared with a GAPDH control, without significant variation (data not shown).

In Situ Hybridization to Normal Tissues
In order to assess the localization of H-RYK mRNA, in situ hybridization was performed on a

panel of adult and fetal human tissues. In the colon, there was a low intensity of signal in the stroma (Fig. 4 a and b). Surface epithelium also showed some expression. In the breast, the signal was primarily localized to mammary epithelium (Fig. 4c). In the lung (fetal) the mRNA signal was observed in the epithelium of devel-
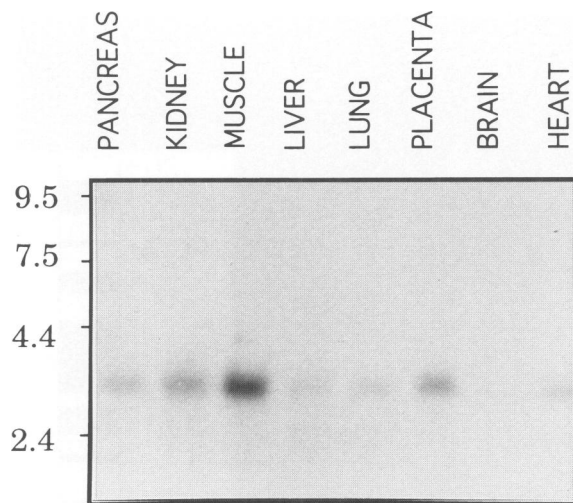
FIG. 3. Northern blot, poly(A)⁺RNA from adult tissues (Clonetech) hybridized to RTK15 PCR fragment

A single transcript of 3.0 kb was expressed. The autoradiogram was exposed for 5 days at −70°C. Size markers (kb) are shown. Longer exposure showed a faint signal in brain.

oping alveoli. It was also present in lower intensity in the cuff of smooth muscles and epithelium of larger airways (Fig. 4d). H-RYK mRNA localized to the outer cortical layers of the fetal brain, with lesser amounts within the inner layers (Fig. 4e). In the fetal kidney, a high level of expression was observed in the neogenic zone of the cortex, but declined rapidly in the medulla (Fig. 4f). No definite signal was seen in the tubules. In the normal ovary, no expression was observed in the epithelium or stroma. Overall, H-RYK mRNA was localized to both the epithelial and mesenchymal compartments in the different tissues examined, in a pattern distinctive for each tissue.

In Situ Hybridization of Ovarian Tumors

In benign ovarian tumors, there was minimal amount of H-RYK mRNA, usually in the stroma (Fig. 5a). In borderline tumors of the ovary, the stromal expression was again more obvious than epithelial expression (Fig. 5b). In contrast, in malignant epithelial tumors, the mRNA levels were markedly increased compared to benign or borderline tumors (Fig. 5c). The localization of H-RYK mRNA was predominantly confined to the malignant epithelium, with some stromal signal evident in individual tumors. Adjacent

normal epithelium expressed relatively low levels of mRNA. A panel of 25 malignant ovarian tumors of differing histology and grade were examined for H-RYK mRNA (Table 1). The data show that there is variation in levels, though H-RYK is predominantly expressed in the malignant epithelial cells, with minimal to absent levels in the stroma.

Biochemical Characterization

In order to characterize the protein expressed by H-RYK, polyclonal antisera were raised in rabbits against synthetic peptides conjugated to thyroglobulin (15.1 and 15.2, corresponding to amino acid 321–337 and 552–568, respectively). The antisera were affinity purified against the cognate peptides. and used to identify the protein encoded by H-RYK by Western blotting in 41M cells, an ovarian cancer cell line. Two bands of equal intensity were identified migrating around 100 kD by 15.1 and 15.2, and blocked by cognate peptide (Fig. 6a). The two bands detected may represent two isoforms of H-RYK. Another protein of around 45 kD was detected by the two antibodies on longer exposure (Fig. 6b). The H-RYK cDNA was cloned into an expression vector pcDNA1 and CHO cells were transiently transfected. An identical protein of 100 kD was detected by the antisera in transfected cells, compared with the vector control transfected cells (Fig. 6c). Thus, H-RYK encodes for an approximately 100 kD protein, and there maybe two isoforms, that maybe cleaved to form smaller proteins of 45 kD. To evaluate the catalytic activity of H-RYK, immune complex in vitro kinase assays were performed using 15.2 antiserum in 41M cells in the presence of ^{32}P-ATP and Mn^{++}. No evidence of autophosphorylation of H-RYK was detected in spite of varying experimental conditions, such as cations, addition of vanadate, or amount of cells, while positive control receptor tyrosine kinases (EDDR1 and EGFR receptor) showed autophosphorylation. This data was confirmed by blotting with antiphosphotyrosine antibody following immunoprecipitation in the presence or absence of vanadate, using either antisera. No detectable phosphorylation was observed even upon transfection of H-RYK cDNA into CHO cells.

To analyze the expression of H-RYK further, a panel of cell lines (malignant and nonmalignant) from different tissues were examined using both antisera (Fig. 7). Ovarian cell lines 41M, OAW28, UC101, PE06, and SKOV-3 expressed
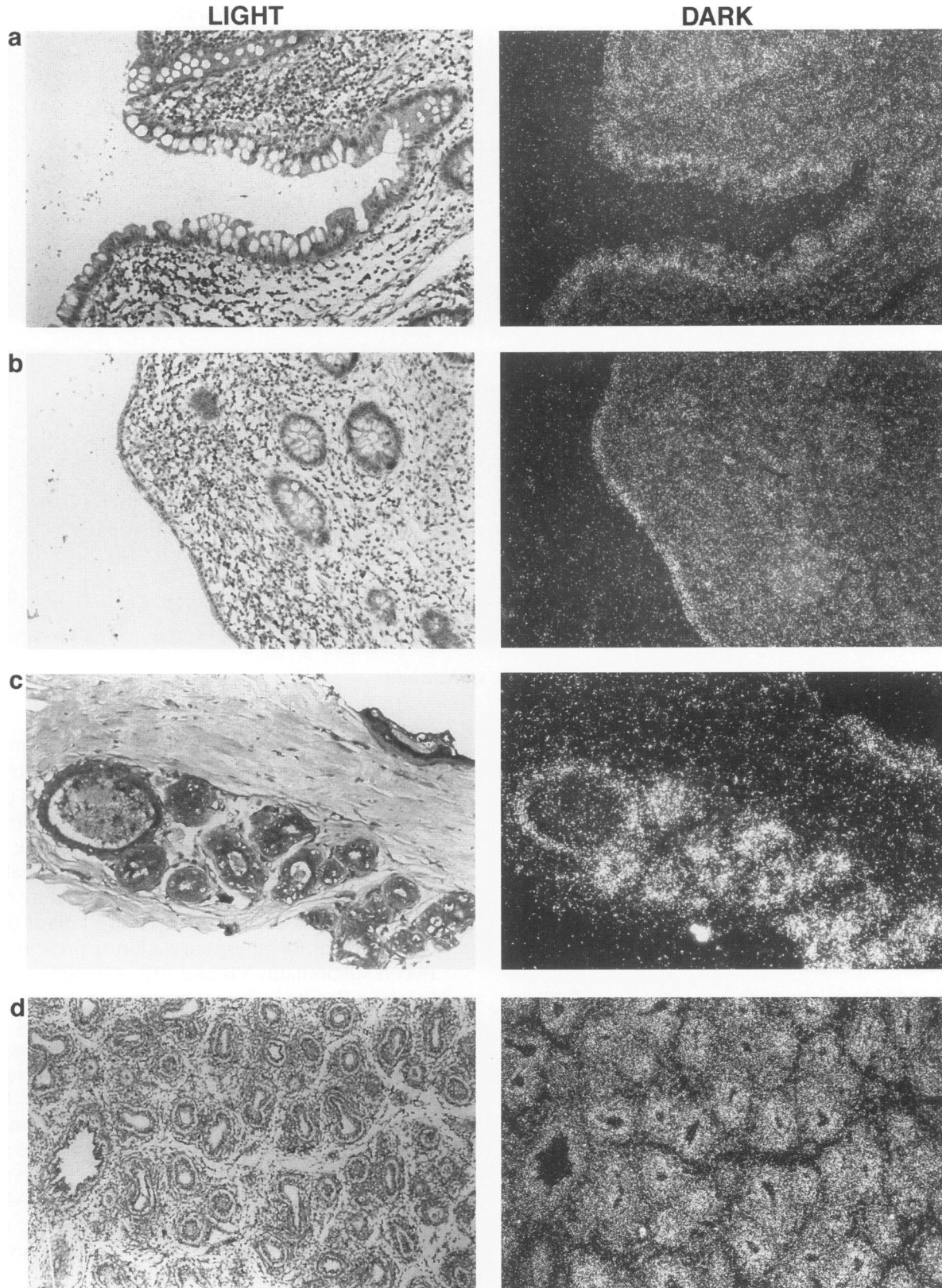
FIG. 4.   In situ hybridization of a panel of human tissues using a ^{35}S-labeled antisense riboprobe (clone 15.5BX3)

Each pair of photograph shows both light and dark fields. (a and b) Colon. (c) Breast. (d) Fetal lung. (e) fetal brain. (f) fetal kidney. The dark fields show localization of message to the epithelial and stromal cells. Magnification before photography was between 10× and 20×.
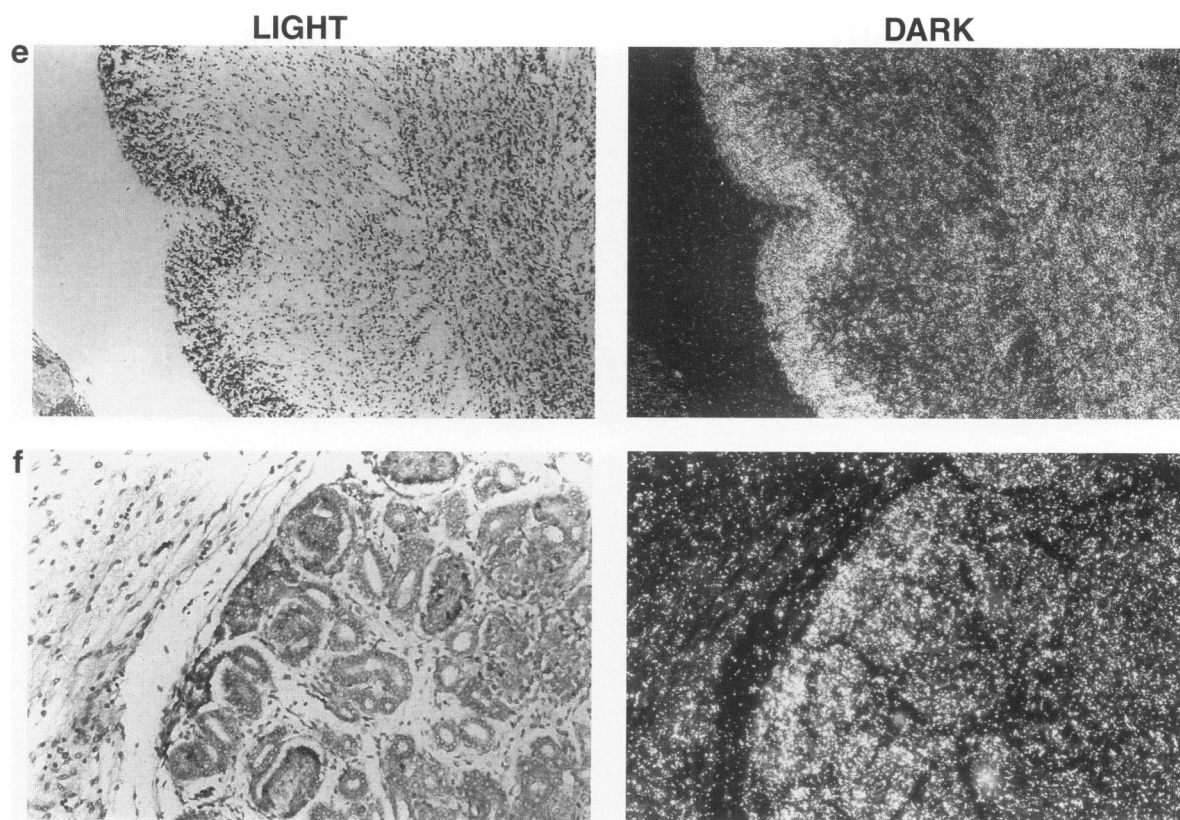
FIG. 4. Continued

the H-RYK protein. Daudi, HL60, K562 (hemo-poietic cell lines) as well as 293 (fetal kidney), BRL (rat liver), C6 (rat glial tumour), and MDBK (bovine kidney) also expressed the H-RYK protein. Both 15.1 and 15.2 cross-reacted with the mouse RYK protein in NIH3T3 cells.

DISCUSSION

The H-RYK gene represents a new class of RTKs, due to the unique sequence alterations in the catalytic domain (Fig. 1) and the novel extracellular domain. The canonical motif GxGxxG in the subdomain I of the catalytic domain is altered to QxGxxG. This glycine rich loop is important in keeping apart the "N" and "C" terminal lobes of the catalytic domain by steric interactions with the "DFG" triplet in subdomain VII which is conserved among all kinases (29). In the latter motif in H-RYK, the critical aspartate is conserved but the phenylaline and glycine are altered to asparagine and alanine respectively giving the motif "DNA". The aspartate has been shown by in vitro mutagenesis to be important for kinase activity (30). Glycine to alanine is a

conserved substitution, whereas phenylalanine to asparagine is a significant alteration. Structural analysis of the insulin receptor suggests that sequence alterations in these conserved residues may contribute to poor conformation of the activation loop (29). However, the lysine distal to the glycine rich loop which is implicated in ATP binding, is conserved. The tyrosine kinase residue essential for phosphorylation is present between subdomains VII and VIII. An explanation for the difficulty in demonstrating catalytic activity of the receptor may be because of one of the above sequence alterations. A receptor tyrosine kinase "klg" with an extracellular domain similar to the immunoglobulin superfamily and homology to v-*sea* (39%) in the catalytic domain was recently isolated. This receptor also does not demonstrate any catalytic activity, in which the "DFG" triplet has been substituted by "ALS" (31). A detailed comparison of the kinase domains between "klg" and H-RYK failed to reveal any other significant differences.

Recently, it has been shown that peptide motifs surrounding the phosphorylated tyrosine provide unique sites for binding of SH$_2$ domain–containing proteins (27,28). There are four po-

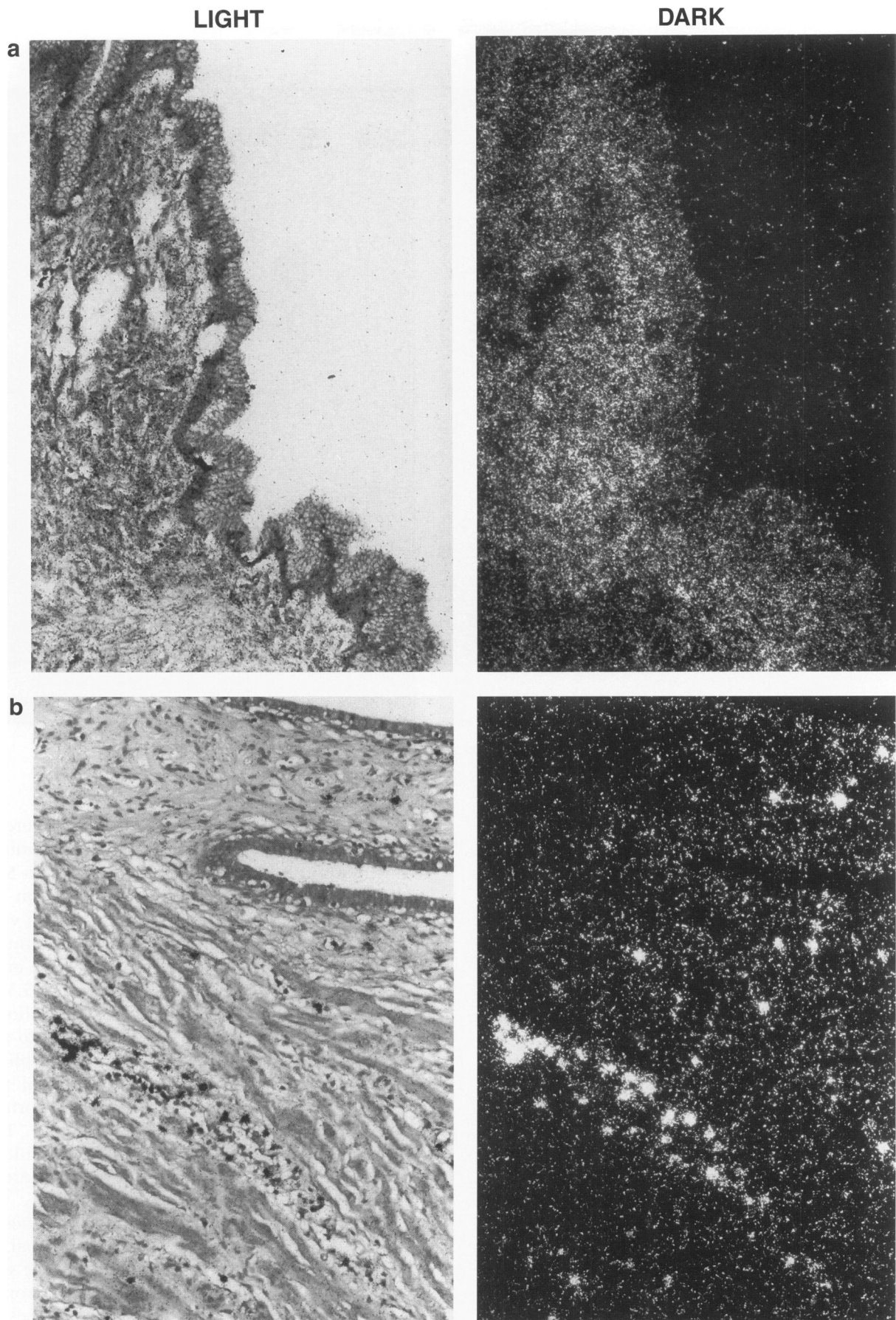LIGHT   DARK

FIG. 5. In situ hybridization of a benign cystadenoma of the ovary (a), borderline tumor of the ovary (b) and well-differentiated serous adenocarcinoma of the ovary (c)
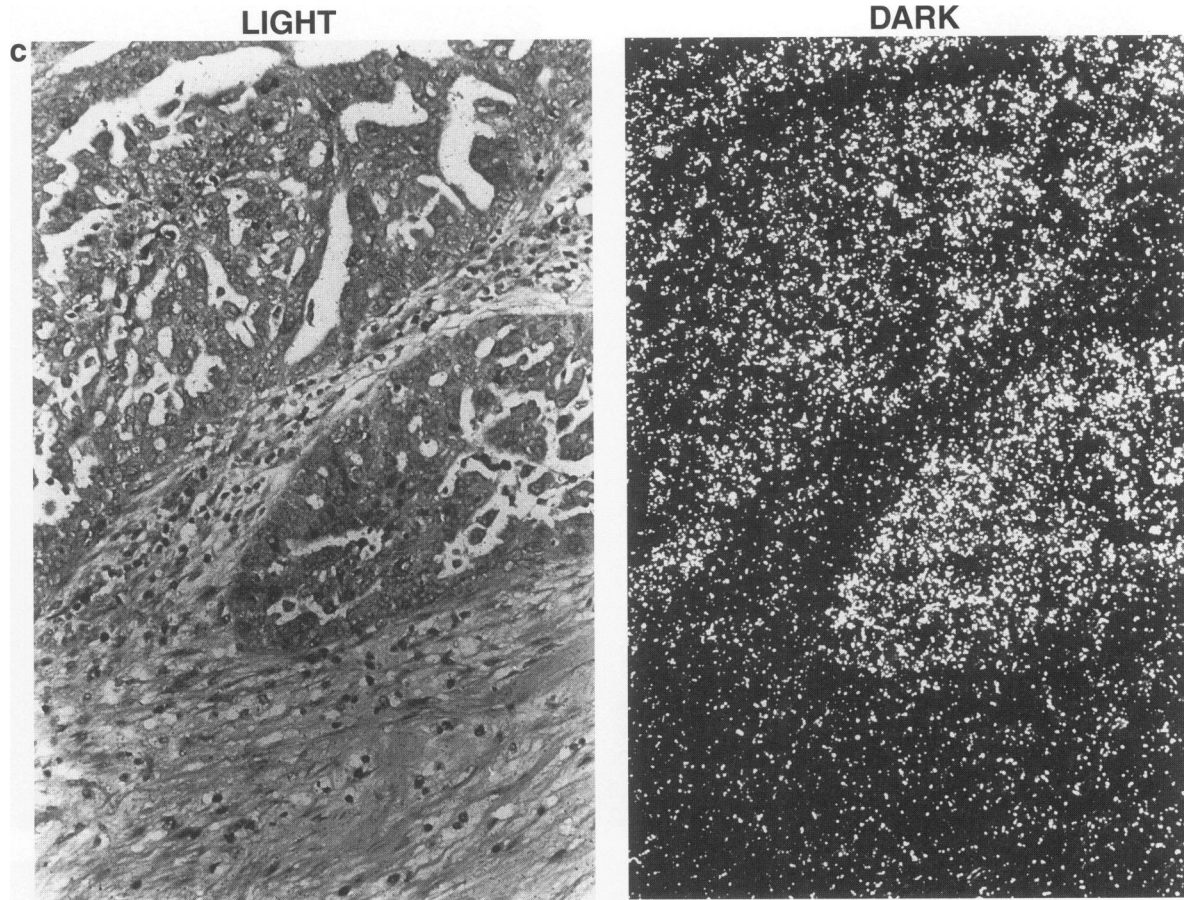
FIG. 5. Continued

tential tyrosine residues within the catalytic domain, with motifs suggestive of probable binding sites to known SH$_2$ domain–containing proteins. Such motifs in other receptors usually occur outside the catalytic domain. This implies that the substrates for H-RYK maybe hitherto uncharacterized proteins or it may signal via an intermediate docking protein like IRS-1, as in the case of the insulin receptor (32). It is possible now to test directly the preferred substrate in vitro, by examining possible phosphorylation sites using techniques described by Songyang (27).

The mRNA for H-RYK is relatively abundant in skeletal muscle, though expressed in all other tissues. Unlike the mouse homolog of H-RYK (33,34), only one transcript of 3.0 kb was observed. At the mRNA level, no significant variation was observed in eight ovarian cancer cell lines. The splice variant encoding an extra three amino acids, serine, leucine, and glycine, is immediately NH$_2$ terminal to a tyrosine in the juxta-membrane domain. This variant was shown to be expressed equally at the mRNA

level, in a panel of ovarian cell lines. There is as yet no evidence of residues amino terminal of the phosphotyrosine being important in SH$_2$ domain interactions. The only exception to this rule, though yet to be demonstrated in vivo is the "NPXY" motif thought to be important in internalization of the LDL receptor, where amino-terminal residues may be critical (35,36). Recently, it has been shown that the phosphotyrosine binding domain (PTB), in contrast to the SH$_2$ binding domain of SHC, binds to a phosphorylated tyrosine within the context of the "NPXY" motif. The asparagine is an important requirement for the binding of SHC (37). The two isoforms of H-RYK produced as a result of this splicing, may thus have different functions although this remains to be shown.

The expression of H-RYK in normal tissues as analyzed by in situ hybridization was unusual. In most of the tissues examined, the message was observed in epithelial cells and in the stroma. The expression in stroma, which comprises of various types of cells, suggests a role in cell-cell

TABLE 1. Expression of H-RYK as determined by in situ hybridization on a panel of ovarian tumors

Name	Histological Type	Grade	Expression[a]	
			Epithelium	Stroma
S.R.[b]	Mucinous adenocarcinoma	—	−	++
U.K.[b]	Mucinous adenocarcinoma	—	−	++
J.T.[b]	Serous adenocarcinoma	—	+/−	+/−
J.G.[b]	Mucinous adenocarcinoma	—	−	−
B.A.[b]	Mucinous adenocarcinoma	—	+	+
A.L.	Endometroid adenocarcinoma	Well differentiated	−	−
D.H.	Mucinous adenocarcinoma	Well differentiated	+/−	+
J.A.	Serous adenocarcinoma	Moderate diff.	+/−	+/−
M.S.	Clear cell adenocarcinoma	Moderate diff.	+	−
B.S.	Endometroid adenocarcinoma	Moderate diff.	+++	+
I.N.S.	Serous adenocarcinoma	Moderate diff.	+++	+
J.C.	Clear cell adenocarcinoma	Moderate diff.	+/−	−
S.B.	Mucinous adenocarcinoma	Moderate diff.	++	+/++
P.J.	Endometroid adenocarcinoma	Moderate/focally Poorly diff.	++	−
M.B.	Endometroid adenocarcinoma	Poorly diff.	+++	+/−
J.S.	Papillary adenocarcinoma	Poorly diff.	++	−
E.M.	Endometroid adenocarcinoma	Poorly diff.	++	+/−
E.K.	Clear cell adenocarcinoma	Poorly diff.	+/−	−
J.N.	Endometroid adenocarcinoma	Poorly diff.	nd	nd
G.C.	Serous adenocarcinoma	Poorly diff.	+++	−
B.J.	Clear cell adenocarcinoma	Poorly diff.	++	
C.B.	Clear cell adenocarcinoma	Poorly diff.	−	−
M.C.	Mucinous adenocarcinoma	Poorly diff.	+	−
B.S.	Serous adenocarcinoma	Poorly diff.	+++	−
J.L.	Serous adenocarcinoma	Poorly diff.	+	−
J.R.	Serous adenocarcinoma	Poorly diff.	+++	−
P.S.	Serous adenocarcinoma	Poorly diff.	+/−	−
M.A.	Serous adenocarcinoma	Poorly diff.	+	−
P.R.	Serous adenocarcinoma	Poorly diff.	++	−

[a]+ and −, presence and absence of H-RYK mRNA, respectively.
[b]Represents borderline tumors of the ovary.
nd, not detected.

interactions. This is suggested further by the presence of leucine rich motifs in the extracellular domain of H-RYK, observed in other cell adhesion molecules.

The pattern of expression in the ovary is interesting. In the normal adult ovary, there was little expression in the normal ovarian epithelium or stroma. In benign and borderline tumors, increased signal strength from the mRNA in the stroma was observed with minimal epithelial expression. In contrast, in malignant epithelial tumors, there was a pronounced increase in expression predominantly localized to the epithelium. Adjacent normal epithelium tissue in sections of malignant ovarian tumors did not show any expression of H-RYK. The explanation for
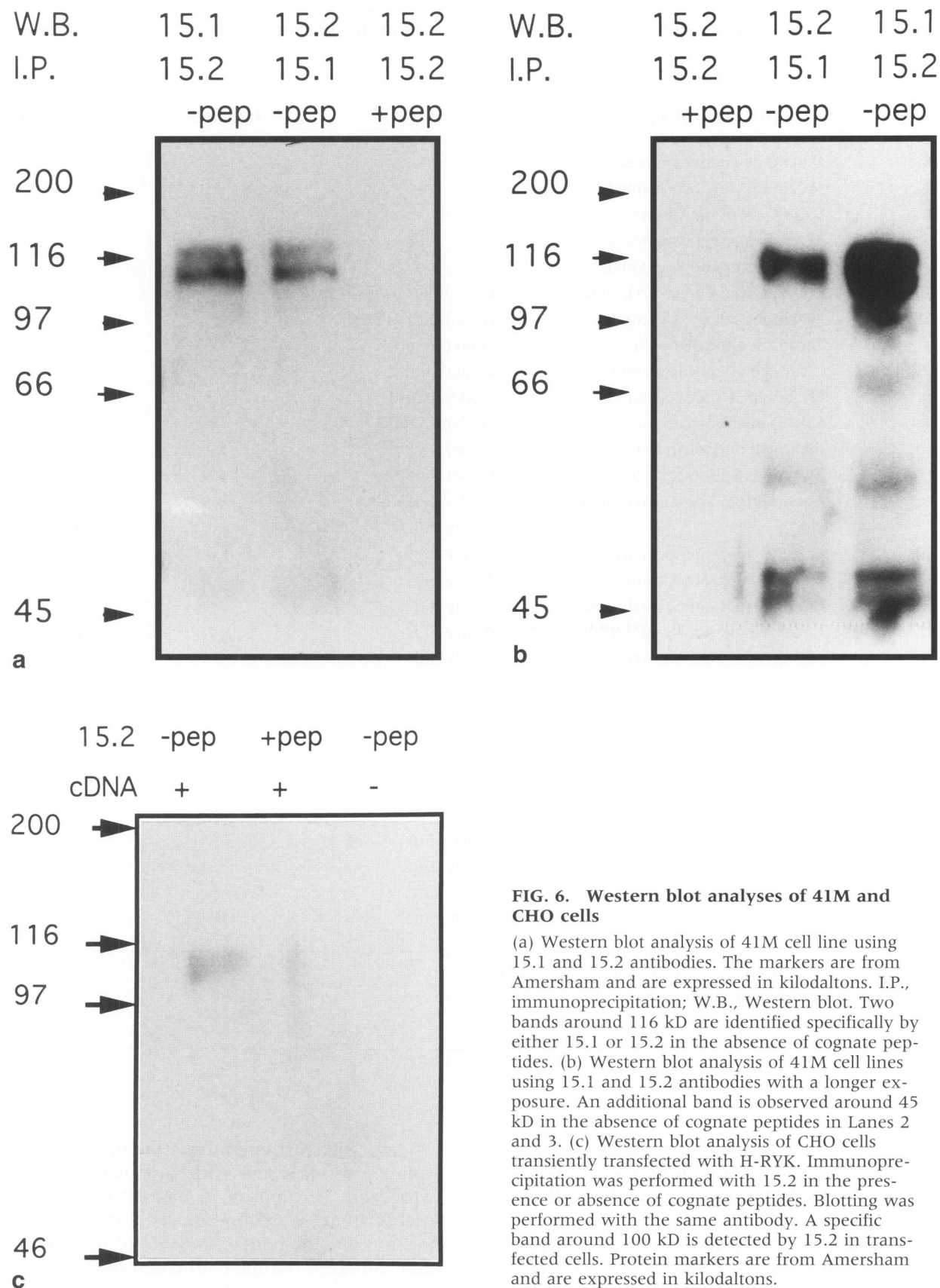
FIG. 6. Western blot analyses of 41M and CHO cells

(a) Western blot analysis of 41M cell line using 15.1 and 15.2 antibodies. The markers are from Amersham and are expressed in kilodaltons. I.P., immunoprecipitation; W.B., Western blot. Two bands around 116 kD are identified specifically by either 15.1 or 15.2 in the absence of cognate peptides. (b) Western blot analysis of 41M cell lines using 15.1 and 15.2 antibodies with a longer exposure. An additional band is observed around 45 kD in the absence of cognate peptides in Lanes 2 and 3. (c) Western blot analysis of CHO cells transiently transfected with H-RYK. Immunoprecipitation was performed with 15.2 in the presence or absence of cognate peptides. Blotting was performed with the same antibody. A specific band around 100 kD is detected by 15.2 in transfected cells. Protein markers are from Amersham and are expressed in kilodaltons.
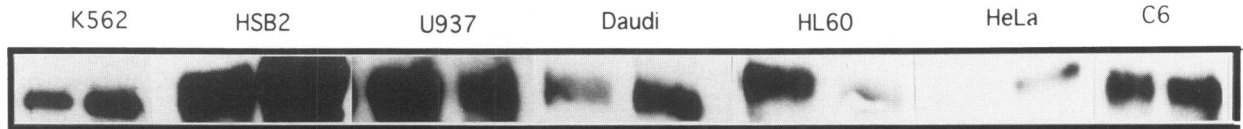
FIG. 7.   A composite photograph of Western blot analysis of cell lines derived from different tissues
The antibody 15.1/15.2 was used in immunoprecipitation and Western blotting. A protein of ≈100 kD was identified in all cell lines examined.

such differential pattern of expression in benign over malignant tumors is at present unclear. Detailed analysis of a panel of malignant ovarian tumors confirmed this observation. Further evaluation of the expression of H-RYK using antibodies will have to be performed to confirm if the levels of H-RYK mRNA result in similar patterns of expression of the protein.

The proteins immunoprecipitated by antisera specific to H-RYK was found to be approximately 100 kD. There were two bands of equal intensity identified in 41M cells, one slightly larger than 116 kD and the smaller one less, suggesting the presence of two isoforms. Careful examination of the gel, revealed another band at approximately 46 kD. This may represent a protein product cleaved from the extracellular domain at the "KRRK" sequence. Alternatively, it may signify an associated protein which is co-immunoprecipitated. The size of the 100-kD protein is larger than that predicted from the cDNA by at least 30–40 kD. It is possible that this is due to glycosylation, as there are at least five potential N-glycoprotein sites in the extracellular domain of H-RYK. In intact 41M cells, no change in mobility was observed following tunicamycin treatment for at least 24 hr, which maybe reflective of the half-life of H-RYK, other than absence of glycosylation. The H-RYK protein is expressed ubiquitously as shown by the results of a screen on a panel of malignant and non-malignant cell lines from different tissues.

Although H-RYK on primary structure has a high degree of homology to RTKs, no catalytic activity could be demonstrated. This maybe due to low basal levels of phosphorylation in the absence of its ligand. Alternatively, the critical changes in the sequence maybe a contributing factor by altering tertiary structure. To investigate the latter, an approach based on construction of a chimeric receptor would be valuable. This has also been informative in assessing the function of the c-erbB3 protein, a member of the

epidermal growth factor receptor (EGFR) family, where it was difficult to demonstrate catalytic activity. Substitution of the extracellular domain of c-erbB3 with that of EGFR showed that the catalytic domain of c-erbB3 was functional when stimulated with EGF (38). However, NIH3T3 cells contain some murine EGF receptors which might have been contributory to the phosphorylation observed in the chimaeric receptor transfectants. More recently it has been shown that heregulin stimulates phosphorylation of c-erbB3 by heterodimerisation with c-erbB2 (39). An approach based on chimaeric receptors will demonstrate whether the catalytic domain of H-RYK can phosphorylate substrates when stimulated with an appropriate ligand. If the catalytic activity cannot be demonstrated, then the putative physiological function of such a receptor and its ligand is intriguing. One possibility as discussed for the "klg" receptor is that of signal attenuation by formation of inactive dimers with other RTKs (31). The increase in expression in malignant ovarian tumors, in such a context, has to be explored further to understand the role of such a receptor in its pathogenesis. The recently isolated Drosophila derailed (drl) gene has been shown to be homologous to the H-RYK gene in the catalytic domain (50%). This gene is functionally important for selection of neuronal pathways (40). It is possible that H-RYK might well have similar functions in mammalian systems.

ACKNOWLEDGMENTS

the Imperial Cancer Research Fund. Mr. R. Katso is a Rosina Valerie Howell Fellow.

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