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CLONING OF THE GENE ENCODING TROP-2, A CELL-SURFACE GLYCOPROTEIN EXPRESSED BY HUMAN CARCINOMAS

Mara FORNARO[1], Roberta DELL' ARCIPRETE[1], Manuela STELLA[1], Cecilia BUCCI[2], Michele NUTINI[1], Maria Grazia CAPRI[1] and Saverio ALBERTI[1,3]

[1]*Istituto di Ricerche Farmacologiche Mario Negri—Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti); and* [2]*Centro di Endocrinologia e Oncologia Sperimentale del CNR e Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli, Napoli, Italy.*

We have cloned by expression the cDNA encoding Trop-2, a cell-surface glycoprotein expressed by most human carcinomas. Formal proof of the identity of the clone is the hybridization to DNA and RNA from genomic *TROP2* transfectants. *TROP2* is a single-copy gene in human cells, hybridizes to a single 1.8-kb mRNA from expressing sources and encodes a 35,709 Da type-I transmembrane protein with a single transmembrane domain. *TROP2* is essentially identical to *GA733-1*. Thus, we have proven that *GA733-1*, for which a protein product had not been identified, is a functional gene. *TROP2* is also homologous to *TROP1/KSA/GA733-2*, confirming the serological similarities between the 2 molecules. The homology between the Trop-1 and Trop-2 peptides is clustered in 2 extracytoplasmic domains and in the transmembrane/cytoplasmic region. Twelve cysteines and a potential cytoplasmic tyrosine phosphorylation site are also conserved. Trop-1 and Trop-2 are homologous to serum IGF-II-binding proteins and appear as signal transducers. Thus, they likely represent novel cell-surface receptors and may play a role in regulating the growth of carcinoma cells. On the other hand, we have found no evidence for a role of Trop-2 and Trop-1 as homophilic adhesion molecules.
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Trop-2 is a monomeric cell-surface glycoprotein expressed at high levels by normal human trophoblast cells and multistratified epithelia and by the majority of human carcinomas (Lipinski *et al.*, 1981; Fradet *et al.*, 1984; Miotti *et al.*, 1987; Alberti *et al.*, 1992). We hypothesize that gp50/Trop-2 may play a role in the growth of normal or transformed cells. We have biochemically characterized this molecule (Alberti *et al.*, 1992) and shown that it is recognized by the T16 (Fradet *et al.*, 1984) and MOv-16 (Miotti *et al.*, 1987) monoclonal antibodies (MAbs). To define the structure of the Trop-2 protein and the regulation of its expression, and to obtain a full-length gene for functional studies, we undertook the cloning of the *TROP2* gene.

MATERIAL AND METHODS

Cells

The human OVCA-432 ovarian carcinoma (Alberti *et al.*, 1992) and BEWO choriocarcinoma cell lines (Alberti and Herzenberg, 1988) were grown in RPMI-1640 medium (GIBCO, Grand Island, NY), supplemented with glutamine, penicillin and streptomycin and 10% FCS (HyClone, Logan, UT). The human 143 B osteosarcoma cell line (ATCC), monkey COS-7 and murine L cells were maintained in DMEM (GIBCO), with 1 g/l glucose and sodium piruvate, supplemented with glutamine, penicillin and streptomycin and 10% FCS (culture medium). L-cell transfectants were maintained in DMEM/HAT culture medium. Human peripheral-blood leukocytes (PBL) were purified from peripheral blood by centrifugation over a Ficoll cushion.

Plasmids

The CDM8 vector (Seed and Aruffo, 1987) was a gift of Dr. B. Seed. The pBJI-neo vector (Lin *et al.*, 1990) was kindly supplied by Dr. M. Davis. The Bluescript vector was obtained from Stratagene (La Jolla, CA).

Antibodies

The GA733 MAb (Herlyn *et al.*, 1984) was kindly supplied by Dr. H. Koprowski (Malvern, PA). The MOv-16 (Miotti *et al.*, 1987) MAb was a gift of Dr. M.I. Colnaghi (Milan, Italy) and the T16 (Fradet *et al.*, 1984) and HT-29/26 (Klein *et al.*, 1990) hybridomas were supplied by Dr. C.E. Klein (Würzburg, Germany). The 162-46.2 anti-Trop-2, 162-21.2 anti-Trop-1 (Lipinski *et al.*, 1981), T16 and HT-29/26 hybridomas were grown as ascites in BALB/c or nude mice. MAbs were purified either by affinity chromatography on protein-A Sepharose or by ion-exchange chromatography essentially as described (Hardy, 1986). Purified antibodies were fluorescein-isothiocyanate (FITC) conjugated as described (Hardy, 1986). Briefly, purified antibody solutions were salt-exchanged to bicarbonate-carbonate buffer, pH 9.4, using a PD-10 column (Pharmacia, Uppsala, Sweden). FITC dissolved in DMSO at 5 μg in 500 μl was added to the antibody in carbonate buffer at 40 μg/mg of antibody. The MAb solution was incubated for 3 hr at room temperature in the dark and the separated from unreacted FITC by salt-exchanging over a PD-10 column equilibrated in saline-TRIS. FITC-goat anti-mouse immunoglobulin (Serotec, Oxford, UK) was used to enhance the fluorescence signal of anti-Trop-2 MAb in the first 2 rounds of selection of the cDNA library. Polyclonal anti-sera recognizing Trop-1 or Trop-2 were obtained by immunizing C3H/HeN mice with cell membranes of L cells, a cell line of C3H origin, transfected with *TROP1* or *TROP2*. Anti-sera were extensively absorbed with untransfected L cells and their specificity was checked by immunofluorescence analysis of several *TROP1*, *TROP2* and control L-cell transfectants.

Flow cytometry immunofluorescence analysis and cell sorting

Cell staining was performed essentially as described (Alberti and Herzenberg, 1988). Briefly, cells were re-suspended in staining medium (SM) at 10^6 cells/ml. SM was 50% HBSS and 50% PBS (GIBCO), supplemented with essential and non-essential amino acids, sodium pyruvate, 0.1% NaN_3 and 3% newborn-calf serum. Complete SM was buffered to pH 7.4 with 20 mM HEPES. Cell suspension (200 μl) was incubated with saturating amounts of the appropriate MAb for 30 min on ice. Cells were washed in SM 3 times and, if necessary, incubated with 1 μg of FITC-anti-serum anti-primary antibody. After 3 washes in SM, the cells were re-suspended in SM with 0.5 μg/ml of propidium iodide to allow gating of dead cells.

[3]To whom correspondence and reprint requests should be sent, at Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy. Fax: (39) 872 578 240.

Abbreviations: FITC, fluorescein-isothiocyanate; MAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBL, peripheral-blood leukocytes; SM, staining medium; SD, standard deviation.

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Fluorescence analysis and sorts were made on fluorescence-activated cell sorters (FACS IV and FACS-STAR, Becton Dickinson, Sunnyvale, CA), used essentially as described by Parks *et al.* (1986). To improve the detection of transfectants stained with FITC-MAb, subtraction of cell autofluorescence (Alberti *et al.*, 1987) and displacement of FITC-stained cells in the red channel (Alberti *et al.*, 1991) were performed as described.

Optical microscopy immunofluorescence analysis

Cells adhering to glass coverslips were washed in SM and incubated with saturating amounts of FITC-MAb for 30 min at 4°C in a moist chamber. The coverslips were then washed and incubated with the FITC-secondary reagent, if necessary. Stained cells were fixed in 3% paraformaldehyde for 30 min at 4°C. Free paraformaldehyde was quenched either by washing in PBS/BSA or by incubating at least for 10 min at room temperature in 50 mM NH_4Cl in PBS. Fixed coverslips were mounted in 10% PBS, pH 8.5, 90% glycerol, 0.1% NaN_3. Samples were analyzed either by conventional immunofluorescence or by confocal immunofluorescence microscopy.

Immunoprecipitation

Immunoprecipitation of live cells after surface labeling with [^{125}I] was performed essentially as described (Mishell and Shiigi, 1981). Briefly, 3×10^6 COS-7 transfectants or control cells were collected in PBS-EDTA and washed in PBS. The cell pellet was re-suspended in 0.5 ml of 10 mM glucose in PBS, and lactoperoxidase, glucose oxidase (Sigma, St. Louis, MO) and 0.5 mCi [^{125}I] were added. The cells were incubated at room temperature for 15 min with gentle swirling. The reaction was stopped by dilution in 5 mM KI. Washed cells were lysed in lysis buffer (0.15 M NaCl, 0.5% NP-40, 10 mMTris, 0.02% NaN_3, 10 mM iodoacetamide, 1 mM PMSF, 1.0 mg/ml pepstatin, 1.0 mg/ml aprotinin, pH 8.0) and passed through a syringe with a 22 G needle. Nuclei were removed by centrifugation at 600*g* for 10 min. The supernatant was pre-cleared with protein A-Sepharose, then incubated at 4°C overnight with protein A-Sepharose conjugated with 5 μg of secondary rabbit anti-mouse Ab/50 μl of beads and 1 μg of specific antibody. After extensive washes, the protein A-Sepharose pellets were re-suspended in 100 μl Laemmli sample buffer, boiled, and analyzed by polyacrylamide gel electrophoresis (PAGE).

DNA transfection

The calcium phosphate co-precipitation technique was followed (Alberti and Herzenberg, 1988; Sambrook *et al.*, 1989). Briefly, 10^6 COS-7 cells in log-phase growth were seeded in each 10-cm-diameter dish (Nunclon, Nunc, Karestrup, Denmark) 8 hr before the transfection. Carrier genomic DNA (10 μg) and 10 μg of plasmid or library DNA per dish were co-precipitated in calcium phosphate. Genomic DNA was purified in guanidinium thiocyanate and banded in CsCl (Alberti and Fornaro, 1990) and plasmid DNA was banded twice in CsCl or extracted with Qiagen (Chatsworth, CA) columns. The DNA precipitates were added to the cells after a wash in complete culture medium and the dishes were incubated at 37°C. After 24 hr the transfected dishes were extensively washed and further incubated for 24 to 48 hr before analysis or sort. For stable transfections, 10^6 L TK$^-$ cells in log-phase growth were seeded in each dish. Transfections were performed either with 10 μg of genomic human DNA co-precipitated with 1 μg of plasmid containing the TK gene or with 10 μg of carrier genomic DNA and 10 μg of the *TROP2* constructs in pBJI-neo or CDM8. Human DNA was extracted from the BEWO choriocarcinoma cell line or from the JM T lymphoma line or PBL (Alberti *et al.*, 1994). Culture in HAT medium or geneticin selected the transfected cells. After HAT selection, *TROP2*-expressing cells were selected by multiple rounds of sorting by flow cytometry. *TROP2* secondary and tertiary transfectants were obtained by transfection of genomic DNA from, respectively, primary or secondary transfectants and selecting both for HAT resistance and for *TROP2* expression. Transfectant names indicate how they were selected. The source of the transfected DNA (JM, BEWO or P for PBL) is indicated first, followed by the number or letter identifying each transfection. The antigen selected (T1, Trop-1; T2, Trop-2) comes next, followed by a dot and the number of the first- and second-round clones, where appropriate. For example, JMNT1.4.17 is a clone obtained from L cells of plate N transfected with DNA from JM cells, selected for Trop-1 expression, cloned a first time (clone number 4) and re-cloned (clone number 17). 2× or 3× indicate secondary or tertiary transfectants respectively.

cDNA and genomic cloning

mRNA for cDNA synthesis was extracted from OVCA-432 ovarian-carcinoma cells and purified over poly-T columns. The cDNA synthesis was primed by poly-T oligonucleotides, the cDNA was size-selected and the fraction larger than 2 kb was used for construction of the cDNA expression library, essentially following the method of Seed and Aruffo (1987). After ligation into BstxI-cut CDM8 vector, the library was electroporated into MC1061/P3 bacteria. Expression screening was performed following the method of Seed and Aruffo (1987), with the following modifications. The library plasmid DNA was banded in cesium-chloride gradients and COS-7 cells were transfected by co-precipitating genomic carrier DNA and library plasmid DNA. Two days after the transfection, *TROP2*-expressing COS-7 cells were selected by flow cytometry with FITC-162-46.2 MAb. Since expressing cells contain the transfected construct as an episome, we sorted *TROP2* transfectants directly in Hirt lysis buffer, *i.e.*, 0.6% SDS, 10 mM EDTA, 10 mM TRIS, pH 7.9. The transfected gene was recovered by Hirt extraction. Briefly, NaCl was added to the Hirt lysate to 1 M. final concentration. The lysate was incubated for 48 hr at 4°C and centrifuged at 200,000*g* for 30 min at 4°C. The supernatant was extracted in phenol and chloroform and precipitated in ammonium acetate/ethanol. After spinning, the pellet was re-suspended in TE and used to transform bacteria. DNA extracted from bacterial colonies obtained from Hirt extracts was used for a further round of transfection and selection of COS-7 cells. A candidate cDNA was isolated from a pool of 6.5×10^4 clones after 2 rounds of expression screening and 2 rounds of sib selection. Sib selection was performed by progressive sub-divisions of the pools of independent *TROP2* candidate clones. Each larger pool was analyzed separately, and positive pools were subdivided into smaller pools and analyzed again, until a positive *TROP2* clone was identified.

The genomic *TROP2* gene was cloned from a human genomic library kindly supplied by Dr. M. Introna, which was constructed in EMBL-4 vector and propagated in K308 *E. coli* cells. Library plating, filter preparation and hybridization were performed using conventional procedures (Sambrook *et al.*, 1989). Replica filters were hybridized with the *TROP2* cDNA at high stringency.

Southern- and Northern-blot analysis

DNA for Southern hybridization and RNA for Northern hybridization were prepared and used essentially as described (Sambrook *et al.*, 1989), as were the DNA probes. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Boehringer (Mannheim, Germany).

DNA sequencing

DNA sequencing was performed following the method of Sanger (Sambrook *et al.*, 1989) using Sequenase kits (USB, Cleveland, OH). Spurious stops and compressions were solved using Taq polymerase (USB) and 7-deaza-dGTP or terminal deoxynucleotidyl transferase (Boehringer). Sequencing tem-

plates were prepared by staggered deletions of the *TROP2* gene sub-cloned in both orientations in Bluescript (Stratagene). Deletions were obtained by titrated ExoIII exonuclease digestion followed by end flushing with S1 nuclease, using the Erase-a-base kit from Promega. The genomic *TROP2* gene was sequenced using internal primers designed following the *TROP2* cDNA sequence or the sequence of the 5' and 3' ends of *TROP2* in Bluescript.

Computer sequence analysis

DNA sequences were analyzed on a VAX 6410 computer using Genetics Computer Group programs (Devereux *et al.*, 1984) and Genbank and EMBL (Heidelberg, Germany) sequence databanks. We searched sequence databanks with the program FASTA. Hydrophobicity plot analysis was performed with the program PEPPLOT. The probability of alpha-helix formation was calculated with the programs PEPPLOT and PLOTSTRUCTURE. Trop-2 and Trop-1 peptide sequences were linearly compared with the program GAP and dot-plot analysis was performed with the COMPARE and DOTPLOT programs. Stringency of the latter was 24 residues on a window of 30. The tyrosine phosphorylation consensus sequences were identified by comparison with published sequences (Cooper *et al.*, 1984) and using the PROSITE databank.

Cell-adhesion assays

To test whether Trop-1 and/or Trop-2 play a role as homophilic adhesion molecules (Litvinov *et al.*, 1994), L cells were transfected with either *TROP1* or *TROP2* in the pBJI-neo expression vector (Lin *et al.*, 1990) or with vector alone. Transfectants were selected for high levels of expression by flow cytometry as described (Alberti *et al.*, 1994). Available clones (Alberti *et al.*, 1994) were tested in preliminary experiments, but were not extensively used due to the variability between clones. For the adhesion assay (Nagafuchi *et al.*, 1987; Nose *et al.*, 1988; Litvinov *et al.*, 1994), bulk transfectants were collected in PBS 1 mM EDTA, filtered through 20-μm pore nylon mesh (Applied Cytometry Systems) to remove cell aggregates, then centrifuged and re-suspended in culture medium. Absence of cell aggregates at this stage of the assay was confirmed by seeding transfectants in control wells before incubation at 37°C in the shaker. Trypsin was avoided, since even if Trop-1 and Trop-2 are resistant to protease treatments (Schön *et al.*, 1993; Litvinov *et al.*, 1994; and data not shown), a brief treatment with trypsin can induce L-cell aggregation (data not shown). DNAse (50 μg/ml) was added to all the incubation buffers, to eliminate spurious aggregation due to release of DNA from dead cells. In all experiments, cell viability was checked by Trypan-blue-dye exclusion and the experiment discarded if any one group's viability fell below 90% at the end of the assay. One millilitre of 6×10^4 cells/ml per experimental group was incubated in sealed 50-ml polypropylene tubes (Falcon, Oxnard, CA) for 1 hr at 37°C in a rotary shaker at 80 rpm (Nose *et al.*, 1988; Litvinov *et al.*, 1994). Cells do not adhere to polypropylene, thus we could avoid the use of agarose (Litvinov *et al.*, 1994) in the adhesion assay. At the end of the incubation period cells were carefully seeded in separate wells in 24-well plates. Since accurate quantitation of the number of cells in each aggregate is extremely difficult in suspension, cells were allowed to adhere to the tissue-culture wells or to microscope glass coverslips for a few hours or overnight before counting. Quantitation was performed by analyzing the tissue-culture wells through an inverted microscope. Duplicate wells were analyzed using a 20× objective. Five random fields were scanned in each well. Alternatively, antibody-stained transfectants on coverslips were observed by immunofluorescence microscopy after fixation in 3% paraformaldehyde in PBS. To try to inhibit a potential homophilic function of Trop-1 or Trop-1, we added to parallel groups of cells a mixture of antibodies directed against each transfected molecule. Since it was not known whether any of the antibodies available efficiently shielded functionally relevant domains of Trop-1 and Trop-2, we used several MAbs together with polyclonal mouse anti-sera anti-Trop-1 or anti-Trop-2. More in detail, Trop-2 transfectants were incubated with 162-46.2, T16 and polyclonal anti-Trop-2 mouse anti-sera. Trop-1 transfectants were incubated with 162-21.2, HT-29/26, GA733 and polyclonal anti-Trop-1 mouse anti-sera. One microlitre of polyclonal anti-sera and a total of 1.5 μg of MAbs were added to each group of 6×10^4 cells. Flow-cytometry analysis indicated that the amount of antibody added was at least 10-fold higher than the minimum saturating amount for the transfectants chosen. For homophilic sorting experiments (Nagafuchi *et al.*, 1987; Nose *et al.*, 1988; Litvinov *et al.*, 1994), TROP1, TROP2 or control L-cell transfectants were mixed together before incubation. After 1 hr at 37°C in a rotary shaker at 80 rpm, cells were seeded and stained as above. The adhesion experiments were repeated at least 4 times per experimental group. Results are presented as mean ± standard deviation (SD) (Table I). As a second approach we used the procedure of Norment *et al.* (1988). Briefly, cell-cell binding was assayed in flat-bottomed 96-well plates in PBS with Ca^{++} and Mg^{++} with 10% FCS. L-cell transfectants were labeled by [^{3}H]-thymidine incorporation. Labeled *TROP1*, *TROP2* or control transfectants were added at 5×10^3 cells/well to wells containing confluent monolayers of unlabeled transfectants; 5 replicate wells were assayed per group. After 1 hr of incubation at 37°C, all wells were gently washed 3 times. After the washes the cells in the wells were lysed and counted by scintillation.

RESULTS

TROP2 *cDNA cloning*

To clone the *TROP2* cDNA, we modified the expression cloning method developed by Seed and Aruffo (1987), as described above, to increase the efficiency of transfection of COS-7 cells and of selections of COS-7 transfectants. We constructed a cDNA library in the CDM8 vector by poly-T priming poly-A$^+$ mRNA from the OVCA-432 cell line, which expresses gp50/Trop-2 at high levels. We transfected the library in COS-7 cells and selected *TROP2* transfectants by flow cytometry. The transfected plasmid was recovered from Hirt extracts of the sorted cells. Two cycles of selection by expression and 2 cycles of purification by sib-selection led to the cloning of the *TROP2* gene. The cloning of *TROP2* was confirmed by immunofluorescence (Fig. 1) and immunoprecipitation (Fig. 2) of transfected COS-7 and L cells (Fig. 3). The staining of *TROP2*-transfected COS-7 cells by FITC-anti-Trop-2 MAbs, *i.e.*, 162-46.2, T16 and MOv16, was specifically blocked by the respective unconjugated MAb, proving the specificity of the binding. No comparable staining of *TROP2*-

TABLE I – AGGREGATION OF CELLS TRANSFECTED WITH TROP-1 OR TROP-2

Transfectants	Single cells[1]	2–6 cells	7–15 cells	≥ 16 cells
L-vector	9.0 ± 5.0	2.0 ± 1.7	1.0 ± 0.6	0.6 ± 0.3
L-Trop-1 + anti-Trop-1 Ab	11.0 ± 4.0	2.0 ± 1.2	1.2 ± 0.5	0.7 ± 0.5
L-Trop-1	9.0 ± 3.0	2.7 ± 0.7	1.1 ± 1.0	0.7 ± 0.3
L-Trop-2 + anti-Trop-2 Ab	5.1 ± 6.0	1.5 ± 1.7	1.1 ± 1.2	0.7 ± 0.3
L-Trop-2	6.0 ± 5.0	1.0 ± 1.0	0.7 ± 0.8	0.9 ± 0.1

[1]Results are expressed as mean ± SD of the cells or aggregates observed in a 20× objective microscopy field after incubation at 37°C and adhesion to tissue-culture plates.

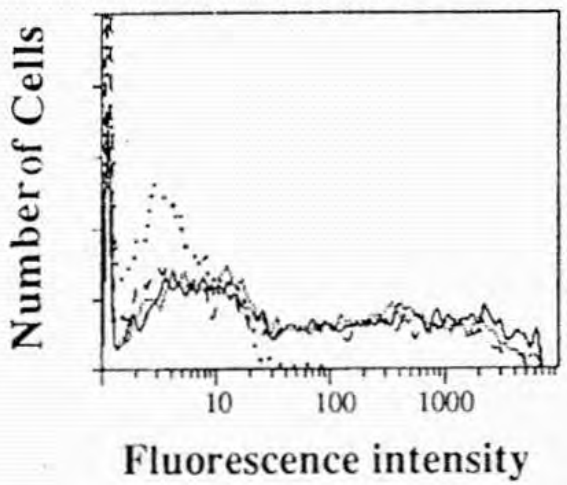

FIGURE 1 – Immunofluorescence analysis of COS-7 cells transfected with the *TROP2* cDNA. Transfectants were stained with fluorescein-conjugated 162-46.2 (solid line), MOv-16 (finely dotted line), T16 (dashed line) or control MAb (dotted line).

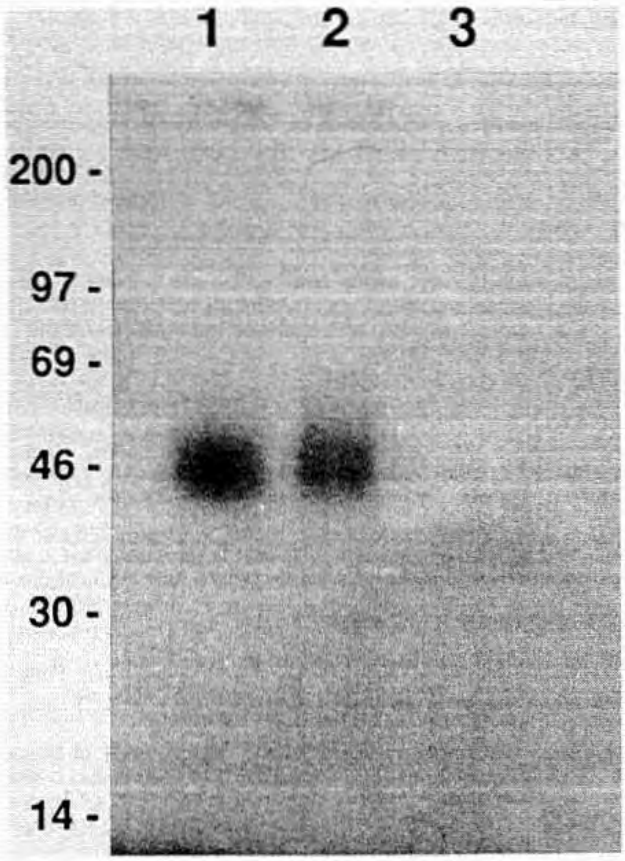

FIGURE 2 – Immunoprecipitation of the Trop-2 protein from *TROP2* COS-7 transfectants after surface labeling with [^{125}I]. Lanes 1 and 2, 2 independent *TROP2* transfectants; lane 3, untransfected COS-7 cells. The immunoprecipitation was performed with the T16 anti-Trop-2 MAb. Samples were analyzed in non-reducing conditions by SDS-PAGE.

transfected COS-7 cells was seen with control FITC-anti-Trop-1 MAbs, i.e. 162-21.2, HT-29/26 and GA733. HT-29/26 and GA733 dimly stain untransfected COS-7 cells, since they

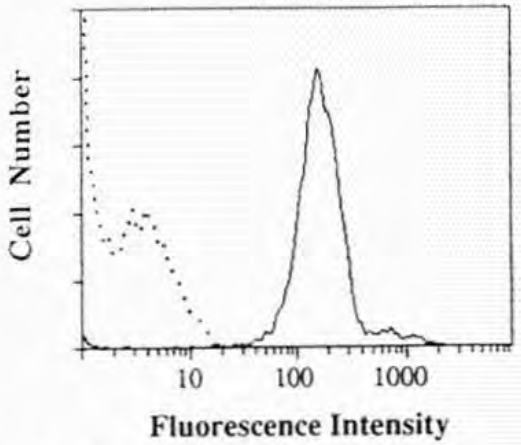

FIGURE 3 – Immunofluorescence analysis of L cells transfected with the *TROP2* genomic clone. Transfectants were stained with fluorescein-conjugated 162-46.2 (solid line) or control MAb (dotted line).

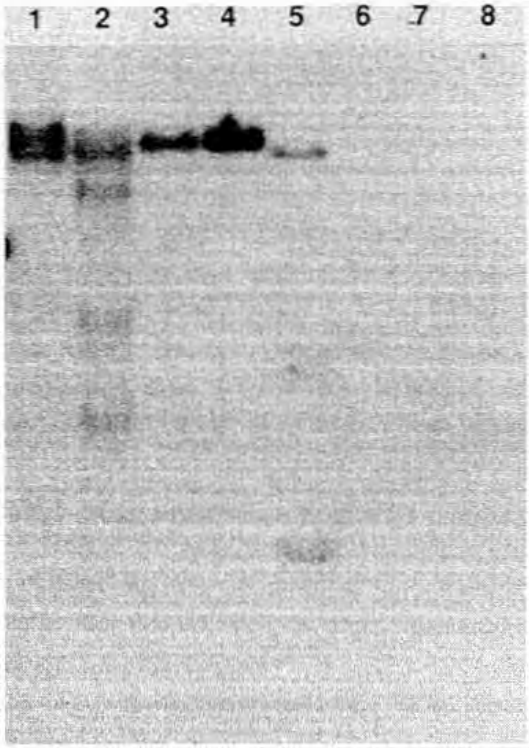

FIGURE 4 – Southern-blot analysis of DNA from *TROP2* transfectants and control cells. *TROP2* transfectants in L cells were obtained after transfection of human genomic DNA. Lane 1, *TROP2* transfectant BEWO11T2.6; lane 2, BEWO11T2.6.1, *i.e.*, sub-clone 1 of BEWO11T2.6; lane 3, 2XT2B, *i.e.*, a secondary *TROP2* transfectant from BEWO11T2.6.1; lane 4, 3XT2A, *i.e.*, a tertiary *TROP2* transfectant from 2XT2B; lane 5, human PBL; lane 6, *TROP1* transfectant JMIT1; lane 7, *TROP1* transfectant JMNT1.4.17; lane 8, unselected transfected L cells. DNA samples were digested with EcoRI. The *TROP2* cDNA was used as a probe.

express low levels of monkey Trop-1, as confirmed by immunoprecipitation (data not shown). *TROP1/KSA/GA733-2* was chosen as a control because it is a cell-surface molecule quite similar to Trop-2 in tissue distribution and biochemical characteristics (Lipinski *et al.*, 1981; Fradet *et al.*, 1984; Miotti *et al.*,

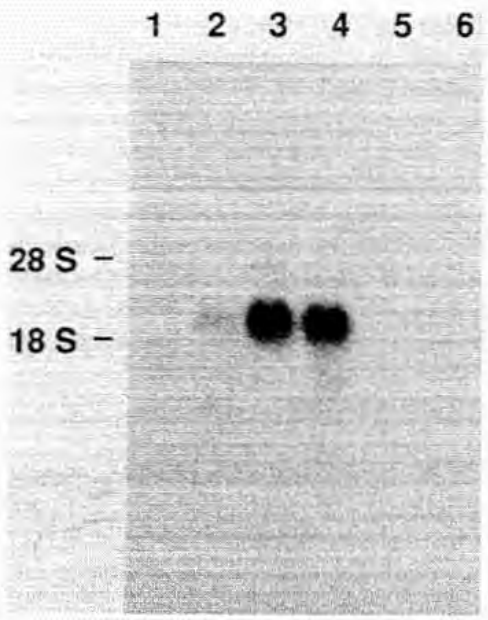

FIGURE 5 – Northern-blot analysis of RNA extracted from *TROP2* transfectants and control cells. *TROP2* transfectants in L cells were obtained after transfection of human genomic DNA. Lane 1, 143 B osteosarcoma cell line; lane 2, BEWO choriocarcinoma cell line; lane 3, OVCA-432 cells; lane 4, *TROP2* transfectant BEWO11T2.6.1; lane 5, *TROP1* transfectant PGT1.5; lane 6, unselected transfected L cells. The *TROP2* cDNA was used as a probe. The distance of migration of the ribosomal 28S and 18S RNAs is indicated.

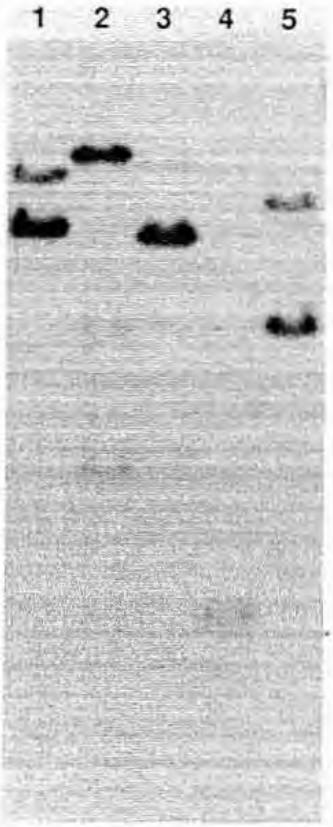

FIGURE 6 – Southern-blot analysis of DNA extracted from human PBL. DNA was digested with lane 1, BamHI; lane 2, EcoRI; lane 3, HindIII; lane 4, PstI; lane 5, BstEII. Filters were hybridized at high stringency with the *TROP2* cDNA.

1987; Alberti *et al.*, 1992; Klein *et al.*, 1990; and see below). Formal proof of the identity of *TROP2* was the hybridization to DNA and RNA from genomic *TROP2* transfectants (Figs. 4, 5), including transfectants that amplify the *TROP2* gene (Alberti *et al.*, 1994) (Fig. 4). Southern-blot analysis of human DNA shows that *TROP2* is a single-copy gene per haploid genome (Fig. 6). Indeed, since *TROP2* is intronless, the pattern of bands predicted by the restriction map fits the observed Southern-blot band patterns both in length and in number. Moreover, the relative intensity of hybridization corresponds to other single-copy genes (*CD5, CD8, TROP1*) when the probes used are of comparable length in identical hybridization/washing conditions (Alberti *et al.*, 1994) (Figs. 4, 6). *TROP2* hybridizes with a single 1.8-kb mRNA species from expressing cells (Fig. 5).

Sequence homology

The *TROP2* cDNA is full length, is almost identical to *GA733-1* over 1,800 bp (Linnenbach *et al.*, 1989) and is 48% similar to *TROP1/KSA/GA733-2* over 1,500 bp (Strnad *et al.*, 1989; Szala *et al.*, 1990; Linnenbach *et al.*, 1993). The 5′ and 3′ ends of the *TROP2* transcription unit are indicated in Figure 7. The 5′ end is 1 bp downstream of the *GA733-1* transcription start site (Linnenbach *et al.*, 1989), while the 3′ end is 2 bp downstream. The open reading frame of *TROP2* encodes a 35,709 Da protein (Fig. 7). Hydrophobicity plot analysis predicts a type-I transmembrane protein with a canonical hydrophobic leader peptide and one transmembrane segment (Fig. 8). The Trop-2 and Trop-1 peptide sequences are 67.3% similar (Fig. 9*a*), confirming previous results (Linnenbach *et al.*, 1993). In particular, 12 cysteines are conserved with essentially perfect alignment in the 2 molecules, suggesting similar folding and overall 3-dimensional structure. High sequence homology is clustered in 2 extracytoplasmic regions and in the transmembrane/cytoplasmic domains (Fig. 9*b*), suggesting an important functional role of these regions. The Trop-1 and Trop-2 cysteine-rich domains contain an EGF-like repeat and a thyroglobulin repeat (Malthiery and Lissitzky, 1987). Trop-2 and Trop-1 possess a potential tyrosine phosphorylation site (Cooper *et al.*, 1984) in their cytoplasmic region, residue 306 and 296 respectively. The amino-acid sequence that follows the tyrosine is predicted to form an α helix and contains 4 acidic residues spaced by 3 or 4 amino acids. Thus, they appear aligned on the same side of the cytoplasmic α helix.

Cloning of the genomic TROP2

To study the regulatory elements of the *TROP2* gene and to define its genomic structure we have cloned the genomic *TROP2*. We hybridized at high stringency a phage human genomic library using the *TROP2* cDNA coding sequence as a probe. Two candidate clones, designed as 14 and 17, were selected from 6×10^5 plaques after 3 rounds of selection. Restriction mapping and partial sequencing indicated that the 2 clones were probably identical, therefore only 17 was analyzed in detail. After digestion of the genomic *TROP2* clone with several enzymes and Southern-blot analysis using the *TROP2* cDNA as a probe, we identified a PvuII/PvuII fragment of 2.8 kb containing the entire coding region. The 2.8-kb fragment was sub-cloned in Bluescript and sequenced entirely (Fig. 7). We also sub-cloned it in the pBJI-neo cDNA expression vector and demonstrated expression after stable (Fig. 3) or transient transfection. Sequence analysis indicates absence of introns in the *TROP2* gene, confirming earlier findings on the *GA733-1* gene (Linnenbach *et al.*, 1989). We also confirmed and extended the similarity with the *GA733-1* gene over 2,200 bp of sequence. The predicted proteins

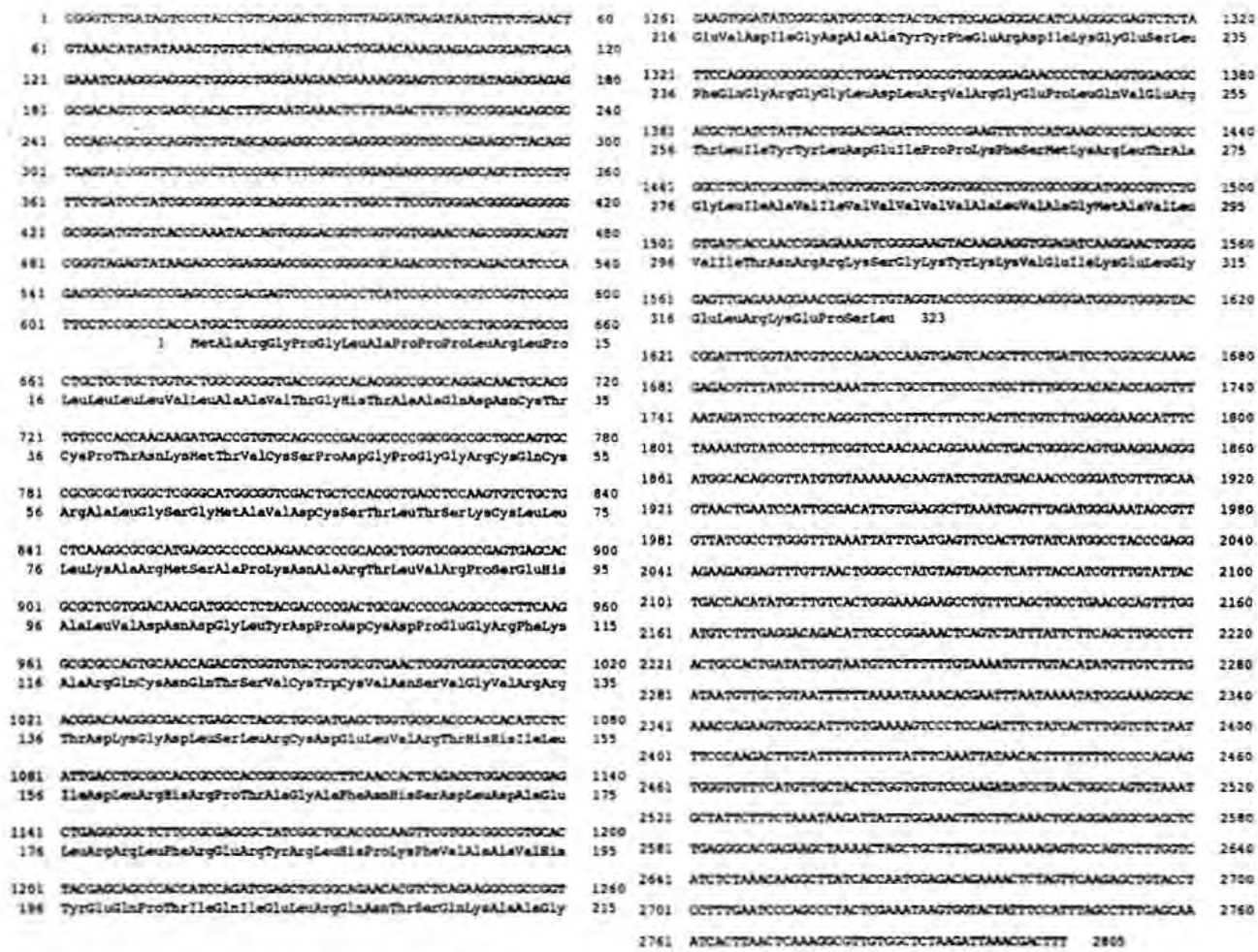

FIGURE 7 – Sequence of the *TROP2* gene. The corresponding translation product is shown. The 5' and 3' ends of the *TROP2* transcription unit, corresponding to bases 547 and 2340, respectively, are underlined. The accession number of the *TROP2* gene is X77753.

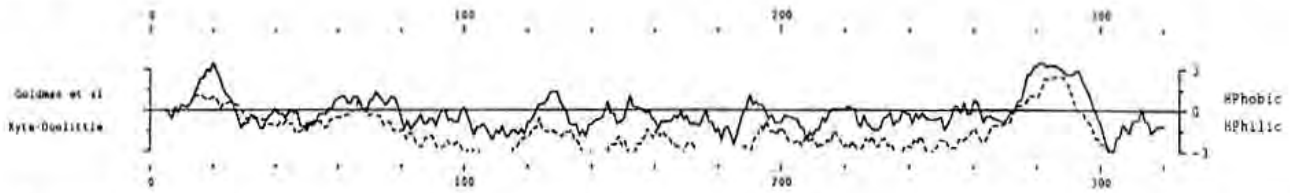

FIGURE 8 – Hydrophobicity plot of the predicted Trop-2 peptide sequence. Two hydrophobic regions, from residue 10 to 25 and from 270 to 295, correspond to the C-terminal end of a hydrophobic leader sequence and to the transmembrane domain respectively.

encoded by the *TROP2* cDNA, the *TROP2* genomic clone and the *GA733-1* gene (Linnenbach *et al.*, 1989) differ at 2 residues. In position 147 a glutamic acid is present in the *TROP2* cDNA and genomic *TROP2*, while an aspartic acid is present in *GA733-1*. In position 217 a glutamic acid is present in the genomic *TROP2* and in *GA733-1*, while an aspartic acid is present in the *TROP2* cDNA. Since the 3 clones derive from independent cloning procedures, *i.e.*, the 3 sources of DNA were different, being derived from different individuals, the changes in their sequence indicate that the *TROP2* gene is polymorphic in the human population. However, the changes identified are somewhat conservative and indicate probable high selective pressure on the function of the molecule.

Cell adhesion

Bulk L-cell transfectants expressing high levels of either Trop-1 or Trop-2 were assayed for increased adhesion. Cells treated as described above were seeded in 24-well plates and scored for the presence of aggregates after adhesion to plastic or glass. The results are analytically presented in Table I. We observed cell aggregation after incubation in the 37°C shaker, as opposed to L transfectants seeded immediately before incubation. However, the number of aggregates was not different between different groups of transfectants. Moreover, the presence of antibodies anti-Trop-1 or anti-Trop-2 during the assay did not detectably modify the distribution of aggregates between the different experimental groups. Finally, since

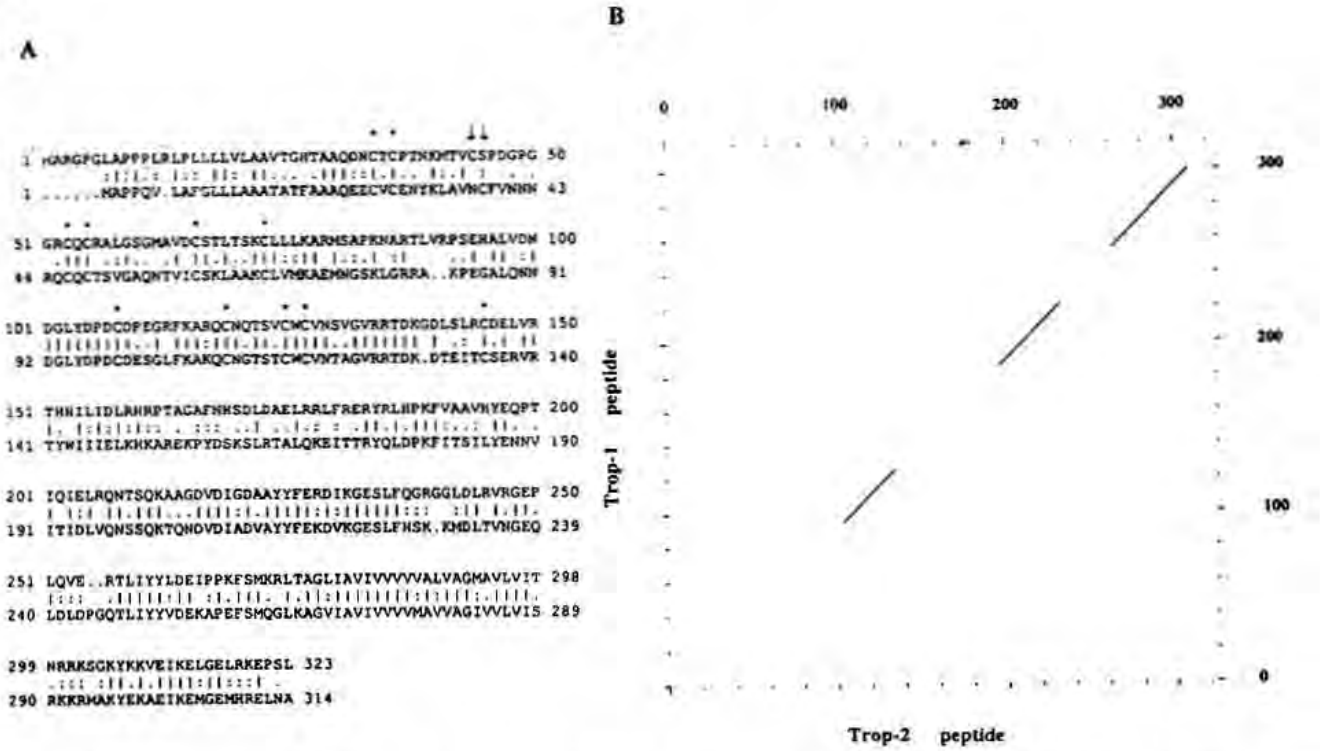

FIGURE 9 – Comparison between the Trop-2 and Trop-1 peptide sequences. *(a)* Gap comparison: top lines, Trop-2; bottom lines, Trop-1. Stars indicate conserved cysteines; vertical arrows indicate a cysteine shifted by one position in the 2 molecules. *(b)* Dot-plot comparison: horizontal axis, Trop-2; vertical axis, Trop-1. Residue numbers are shown.

it was reported that transfectants expressing Trop-1/EGP40 preferentially segregate homophilically when mixed with non-transfected cell populations (Litvinov *et al.*, 1994), we analyzed all the possible combinations of transfectants by immunofluorescence. No skewed distribution of Trop-1 or Trop-2 expression was observed in any experimental group (data not shown). Adhesion assays performed on confluent cell monolayers confirmed these results. Cells adhering to the cells plated in the wells averaged 10 to 20% of the input cells. However, no significant differences between different experimental groups were detected (data not shown).

DISCUSSION

Trop-2 is a 50-kDa Mr cell-surface glycoprotein (Alberti *et al.*, 1992). The gp50/Trop-2 is expressed at high levels by the majority of human carcinomas, even when the epithelial cells of origin show little basal expression of this molecule (Fradet *et al.*, 1984; Miotti *et al.*, 1987). To determine the primary structure of gp50/Trop-2 and to have an expressable construct for functional studies, we have cloned the *TROP2* gene. To clone *TROP2* we used an expression cloning procedure based on transient episomal transfection of a cDNA library in COS-7 cells and flow cytometric selection of expressing cells (Seed and Aruffo, 1987).

The *TROP2* gene is essentially identical over 2,200 bp to *GA733-1* (Linnenbach *et al.*, 1989). Thus, we have proven that *GA733-1*, whose protein product had not been previously identified (Linnenbach *et al.*, 1989), is a functional gene. This also means that Trop-2 is a member of the SCLC-13 cluster, confirming results obtained with the RS7-3G11 and MR54 MAbs (De Leij *et al.*, 1994; Stein *et al.*, 1994). *TROP2* is also 48% similar to *TROP1/KSA/GA733-2* over 1,500 bp (Strnad *et al.*, 1989; Szala *et al.*, 1990), confirming the serological similarities between the 2 molecules. *TROP2/GA733-1* probably originated from a retroposition of *TROP1/GA733-2* (Linnenbach *et al.*, 1993). Indeed, *GA733-1* and *GA733-2* have a different chromosomal location and the latter possesses introns, absent from the former (Linnenbach *et al.*, 1989, 1993). We have confirmed the absence of introns from the genomic *TROP2*. The 5′ and 3′ ends of the *TROP2* transcription unit are indicated in Figure 7. The 5′ end is 1 bp downstream as compared with the *GA733-1* transcription start site, while the 3′ end is 2 bp downstream (Linnenbach *et al.*, 1989). Limited shifts in the transcription start and stop sites are not unusual and do not necessarily indicate the existance of alternative start and stop sites (Lewin, 1994).

The open reading frame of *TROP2* encodes a 35,709 Da protein in good agreement with the Mr of glycosylated Trop-2 (Lipinski *et al.*, 1981). Hydrophobicity plot analysis predicts a type-I transmembrane protein with one transmembrane segment (Lewin, 1994) (Fig. 8). Comparison between the Trop-2 and Trop-1 peptide sequences indicates high similarity of 2 extra-cytoplasmic domains. The essentially perfect conservation of 12 cysteine residues suggests that the 2 molecules probably fold in a similar fashion. The presence of an EGF-like repeat and of a thyroglobulin repeat (Malthiery and Lissitzky, 1987), which is involved in IGF-II binding by serum proteins (Kiefer *et al.*, 1991), is a first indication of a possible role of Trop-2 and Trop-1 as cell-surface receptors. Trop-2 and Trop-1 also possess one potential tyrosine phosphorylation site in their cytoplasmic region (Cooper *et al.*, 1984). The latter is predicted to form an α-helix with 4 acidic residues on one side of it. These characteristics suggest a possible interaction of the cytoplasmic tail with other molecules. Since Trop-2 and Trop-1 do not possess a kinase domain, it would be interesting to determine whether the putative binding protein is a tyrosine kinase. We are now investigating a possible function of Trop-2 and Trop-1 as cell-surface signal transducers. Preliminary

findings indicate that cross-linking either of the 2 molecules with specific MAbs can cause an increase in intracellular calcium levels. Thus, Trop-2 and Trop-1 appear as novel transmembrane receptors, and specific physiological ligands for the 2 molecules are, therefore, likely to exist. A recent article presented the provocative finding that transfectants expressing Trop-1/EGP40 aggregated homophilically and specifically segregated when mixed with untransfected cells (Litvinov *et al.*, 1994). Interesting as these findings are, we find them surprising, since in years of culture of several hundreds of L-cell transfectants expressing either *TROP1* or *TROP2* (Alberti *et al.*, 1994), we have never observed any clear morphological change or growth-pattern modification. Similarly, we have not observed any change in the growth pattern of transiently transfected COS-7 cells. Preliminary experiments set up to replicate the findings of Litvinov *et al.* (1994) indicated large variations in adhesion properties between different clones of L transfectants and large variability between different experiments. Thus, we transfected large polyclonal populations of L cells and identified cell viability and the use of trypsin as critical variables for the assay. As a consequence, we used only EDTA for cell harvesting and added DNAse to all incubation and washing buffers. This protocol revealed marked levels of aggregation of the transfectants. However, we have been unable to observe any contribution of either Trop-1 or Trop-2 to this aggregation. This result is confirmed by the lack of influence of anti-Trop-1 or anti-Trop-2 antibodies on the aggregate formation. Similar results have been obtained by a different approach, following the procedure of adhesion to confluent transfectant monolayers described by Norment *et al.* (1988). The discrepancy between our results and those of Litvinov *et al.* (1994) can be explained in several ways. First, the clearest results of Litvinov *et al.* (1994) have been obtained using L153 mouse epithelial-cell transfectants, whereas those obtained with L cells are much less clear-cut. This may indicate the need for additional epithelial-specific molecules for the *TROP1*-mediated adhesion, and the accessory molecule can in principle be a counter-receptor for *TROP1* and/or *TROP2*. Moreover, it has been our experience that clonal-cell populations, as tested by Litvinov *et al.* (1994), differ widely in adhesion properties, irrespective of the transfected gene. In other terms, they derive from random clonal selection of unknown variables. The revertant clones used by Litvinov *et al.* (1994), *i.e.*, unstable clones that have lost the transfected gene, are in principle good controls for this phenomenon. However, it is possible that differential growth in culture has selected a narrow sub-set of the original cell population, resulting in artefactual cloning or quasi-cloning. The real alternative, in order to obtain results of statistical significance, is to use either large numbers of clones or widely polyclonal populations. The mild treatment with trypsin used to collect the transfectants by Litvinov *et al.* (1994) offers another explanation, apart from the potential to induce aggregation on its own. We have proven that *TROP1/KSA/GA733-2* can undergo proteolytic processing at a single cleavage site (Schön *et al.*, 1993). Trop-2 appears to undergo similar processing (data not shown). Proteolytic cleavage generates a 6-kDa fragment that remains bound to the core molecule through disulphide bridges. The functional role of the cleavage could be the activation of Trop-1 and Trop-2. Trypsin generates a similar or identical cleavage on Trop-1 (Schön *et al.*, 1993). Thus, it might induce activation of Trop-1. Quantitative molecular binding assays are clearly needed to solve this issue.

In summary, since *TROP2* is expressed at high levels by carcinoma cells and appears as a novel cell-surface receptor we hypothesize a crucial role of *TROP2* in the growth and/or development of human cancer cells.

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