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Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis

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The surface receptor for urokinase plasminogen activator (uPAR) has been recognized in recent years as a key molecule in regulating plasminogen mediated extracellular proteolysis. Surface plasminogen activation controls the connections between cells, basement membrane and extracellular matrix, and therefore the capacity of cells to migrate and invade neighboring tissues. We have isolated a 1.4 kb cDNA clone coding for the entire human uPAR. An oligonucleotide synthesized on the basis of the N-terminal sequence of the purified protein was used to screen a cDNA library made from SV40 transformed human fibroblasts [Okayama and Berg (1983) *Mol. Cell Biol.*, 3, 280-289]. The cDNA encodes a protein of 313 amino acids, preceded by a 21 residue signal peptide. A hydrophobicity plot suggests the presence of a membrane spanning domain close to the C-terminus. The cDNA hybridizes to a 1.4 kb mRNA from human cells, a size very close to that of the cloned cDNA. Expression of the uPAR cDNA in mouse cells confirms that the clone is complete and expresses a functional uPA binding protein, located on the cell surface and with properties similar to the human uPAR. Caseinolytic plaque assay, immunofluorescence analysis, direct binding studies and cross-linking experiments show that the transfected mouse LB6 cells specifically bind human uPA, which in turn activates plasminogen. The M_r of the mature human receptor expressed in mouse cells is ~55 000, in accordance with the naturally occurring, highly glycosylated human uPAR. The M_r calculated on the basis of the cDNA sequence, ~35 000, agrees well with that of the deglycosylated receptor.

Key words: extracellular proteolysis/surface receptor/urokinase plasminogen activator

Introduction

Plasminogen activation is involved in processes requiring regulated extracellular proteolysis, like gametogenesis and trophoblast implantation, as well as in pathological conditions in which an aggressive, invasive behavior of cells is noticed, e.g. in tissue destructing diseases and in malignant tumors.

In recent years it has been realized that plasmin formation is the final product of a complex system, involving two types of plasminogen activators, mechanisms for activating plasminogen activator proenzymes, at least two plasminogen activator inhibitors, the substrate plasminogen and specific receptors for plasminogen and plasminogen activator (Reich, 1978; Danø *et al.*, 1985; Blasi *et al.*, 1987; Blasi, 1988; Miles and Plow, 1988). The discovery of a receptor for the urokinase-type plasminogen activator (uPA) (Bajpai and Baker, 1985; Stoppelli *et al.*, 1985; Vassalli *et al.*, 1985) and the study of its properties has highlighted the potential role of the cell surface in this process. Autocrine binding of biosynthetic pro-uPA to the uPA receptors is observed in several cell types, and this process leads to complete saturation of the receptors with the inactive proenzyme (Stoppelli *et al.*, 1986). Receptor bound pro-uPA can be converted to active, two chain uPA by plasmin (Cubellis *et al.*, 1986); the resulting receptor bound uPA is not internalized but remains on the cell surface (Stoppelli *et al.*, 1985, 1986; Vassalli *et al.*, 1985); receptor bound uPA is able to activate plasminogen, and can bind the inhibitor PAI-1 (Vassalli *et al.*, 1985; Cubellis *et al.*, 1989; Stephens *et al.*, 1989). These data outline a surface pathway of plasminogen activation. Cytological studies, in addition, have shown that in tumors receptor bound pro-uPA is present at the invading edges of the tumors (Skriver *et al.*, 1984); at the individual cell level, in both normal and neoplastic cells, uPA is not uniformly located on the cell surface, but rather at contacts between the cell and the substratum in co-localization with vinculin (focal contacts) and between cells (Pöllanen *et al.*, 1987, 1988; Hebert and Baker, 1988). Finally, a surface pathway for formation of plasmin has been demonstrated, involving a receptor bound uPA activity that can be modulated by inhibitors (Cubellis *et al.*, 1989; Ellis *et al.*, 1989; Stephens *et al.*, 1989).

These data support the proposal that cell surface plasminogen activation may give a specific advantage to malignant cells, being at the basis of their invasive and metastatic properties (Stoppelli *et al.*, 1986; Blasi *et al.*, 1987). The role of uPA in invasion and metastasis is well substantiated. Anticatalytic uPA antibodies block or reduce metastasis in two different experimental systems, the Hep2 carcinoma cells in the chicken embryo chorion allantoid membrane (Ossowski and Reich, 1983; Ossowski, 1988a) and the B16 melanoma cells in mice (Hearing *et al.*, 1988). Antibodies and inhibitors of uPA activity inhibit extracellular matrix degradation (Bergman *et al.*, 1986), the invasion of the amniotic membrane (Mignatti *et al.*, 1986) and of a reconstituted basement membrane (Reich *et al.*, 1988). Finally, the involvement of the uPA receptor in the invasion of a healing chicken embryo basement membrane by human carcinoma cells is strongly suggested by experimental data (Ossowski, 1988b).

The central molecule in the surface pathway for plasminogen activation appears, therefore, to be the uPA

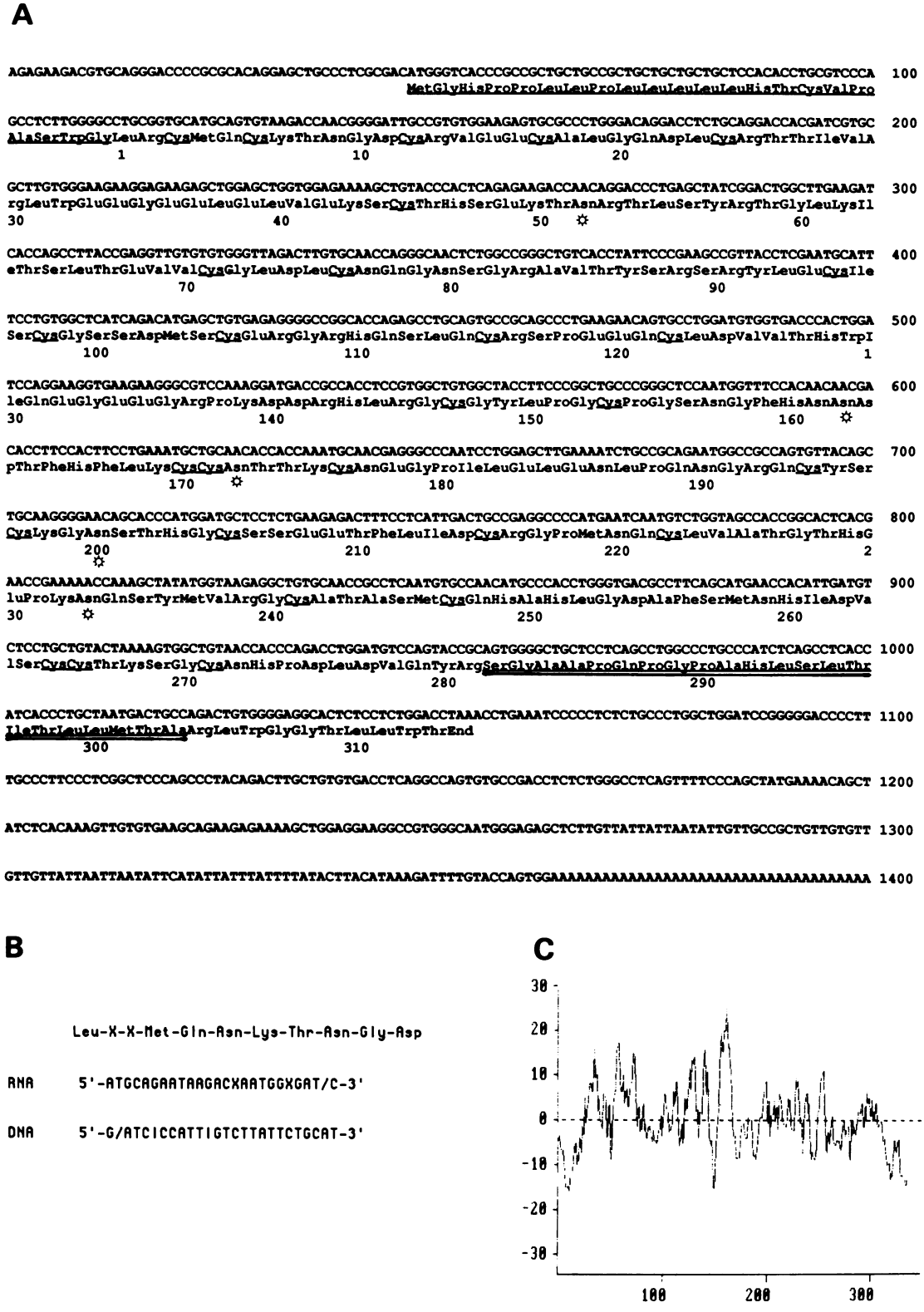

Fig. 1. Structure of the p-uPAR-1 cDNA clone. **Panel A** shows the nucleotide and the translated amino acid sequence of clone p-uPAR-1. The signal peptide is underlined as well as each of the 28 cysteine residues; the putative *trans*-membrane domain is doubly underlined. The star symbols indicate the potential *N*-linked glycosylation sites. **Panel B** shows the initial N-terminal amino acid sequence information and the oligonucleotide synthesized and used for library screening, I stands for inosine. Asn at position 6 had been classified as uncertain due to low yield of the PTH-derivative (Behrendt *et al.*, 1990). **Panel C** shows the hydrophobicity plot. The abscissa shows the amino acid residue position, the ordinate the degree of hydrophobicity calculated using the algorithm of Hopp and Woods (1981) and Kyte and Doolittle (1982).

receptor (uPAR). It is a glycoprotein of ~55 000–60 000 daltons (Nielsen *et al.*, 1988), which recognizes a region of ~12 amino acid residues in the N-terminal growth factor domain of uPA (Appella *et al.*, 1987). It binds not only uPA, but also the proenzyme pro-uPA (Cubellis *et al.*, 1986), the amino-terminal fragment (ATF) (Stoppelli *et al.*, 1985) and

the inactive DFP treated uPA (Bajpai and Baker, 1985), but not the C-terminal, catalytically active, low M_r uPA (Vassalli *et al.*, 1985; Stoppelli *et al.*, 1985). The binding is species specific: mouse receptors do not bind human uPA and *vice versa* (J.-D.Vassalli and D.Belin, personal communication, 1985; Appella *et al.*, 1987; Estreicher *et al.*, 1989). The affinity of uPAR for its ligand is very high, ~0.2 nM and can be regulated by different agents, e.g. phorbol esters and epidermal growth factor (Estreicher *et al.*, 1989; Picone *et al.*, 1989). Both agents increase the number of receptors and decrease their affinity for the ligand.

We now report the cloning, sequencing and expression of the cDNA for the human uPAR.

Results

Isolation and sequence of uPAR cDNA

In order to isolate a uPAR cDNA clone, we have screened a cDNA library constructed from human simian virus 40 (SV40) transformed fibroblasts GM 637 (Okayama and Berg, 1983). This cell line has ~200 000 uPAR/cell which are indistinguishable in binding and cross-linking experiments from those expressed by U937 monocyte-like cells (M.V.Cubellis, data not shown; however, see Figure 4). We have screened the library with an oligonucleotide probe synthesized on the basis of a preliminary N-terminal amino acid sequence obtained by automated Edman degradation of uPAR affinity purified from PMA-treated U937 cells (Behrendt *et al.*, 1990). This tentative N-terminal sequence and the derived complementary oligonucleotide are shown in Figure 1B. The screening conditions were chosen on the basis of the results of hybridization of the oligonucleotide to poly(A)$^+$ RNA isolated from PMA treated U937 cells, in which the synthesis of uPAR is greatly enhanced (Stoppelli *et al.*, 1985; Nielsen *et al.*, 1988; Picone *et al.*, 1989). The library screening was carried out after chloramphenicol amplification and yielded a total of seven clones. An oligonucleotide covering SV40 sequences present in the vector at the 5′ end of the insert was used to determine the sequence at the 5′ end of three clones; these turned out to be identical, including a sequence compatible with that of the oligonucleotide mixture used in the screening. One such clone (p-uPAR-1) was completely sequenced on both strands using subcloning into a pEMBL vector (Dente *et al.*, 1983) and commercial insert flanking or internal synthetic primers. Its sequence is shown in Figure 1A. The cDNA clone is 1363 bp long from the 5′ end to the beginning of the poly(A) stretch. At the 5′ end, 46 nucleotides precede the first ATG codon, which is followed by a 1005 bp sequence with an open reading frame; a nonanucleotide containing two in-frame stop codons is present at the 3′ end of the coding region. The first stop (TAA) codon is separated from the poly(A) sequence by 312 bp of 3′ untranslated sequence. The assignment of the ATG at bp 47 as the translation start site agrees with the consensus for initiating regions (Kozak, 1987). The translated sequence starts with a hydrophobic sequence which conforms to the requirements for a signal peptide (von Heijne, 1986). The putative signal peptide is followed by 313 amino acid residues. Comparison of the sequence shown in Figure 1A with the preliminary N-terminal amino acid sequence (Figure 1B) indicates that the tentative assignment of residue 6 as Asn does not agree with the cDNA derived sequence. However, a definitive N-terminal sequence, which was determined in an independent experiment after preparative electrophoresis, electroblotting and alkylation of the affinity purified protein (Behrendt *et al.*, 1990), assigned residue 6 as Cys. In addition, all 22 amino acid residues which could be determined with certainty in the first 29 amino acid residues of the electroblotted, carboxymethylated protein match the cDNA derived sequence. Thus, although the probe contained a sequence error, it still led to the identification of the right clone. The calculated amino acid composition (not shown) and mol. wt (34 633) agrees well with the data obtained from the purified protein (Behrendt *et al.*, 1990).

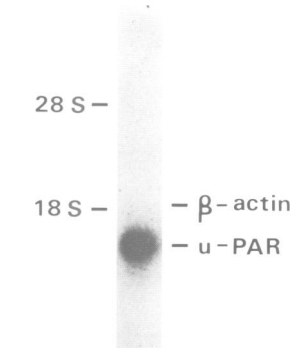

Fig. 2. Northern blot analysis of the poly(A)$^+$ RNA from PMA treated human U937 cells. U937 cells were treated with 100 ng/ml PMA for 24 h and poly(A)$^+$ RNA isolated and elecrophoresed in 1.5% agarose containing formaldehyde. RNA was transferred to nitrocellulose and the filter was hybridized with nick-translated p-uPAR-1 DNA. Position of the ribosomal RNA is indicated, as well as the migration of the β-actin mRNA.

Northern blot analysis of poly(A)$^+$ RNA

To test whether p-uPAR-1 encodes the complete uPAR mRNA, we have carried out two types of experiments. First, we estimated the length of uPAR mRNA by formaldehyde–agarose gel electrophoresis and Northern blot analysis. We isolated poly(A)$^+$ RNA from PMA treated U937 cells since they might contain a higher level of mRNA (Picone *et al.*, 1989). The Northern blot showed a single band which migrated faster than 18S rRNA. Using the migration of 28S and 18S rRNA and of β-actin mRNA as markers, a size of ~1400 nucleotides has been calculated for the uPAR mRNA (Figure 2), which agrees with that of the cloned cDNA.

Expression of p-uPAR-1 in mouse cells

Another indication that the p-uPAR-1 clone encodes a complete cDNA sequence comes from its ability to express functional human uPA receptors in transfected cells. The vector used in p-uPAR-1 cloning contains the SV40 promoter at the 5′ end and polyadenylation and splice sites at the 3′ end (Okayama and Berg, 1983). We have tested the functionality of the p-uPAR-1 clone by transfecting it into mouse LB6 cells and testing transfectants by the caseinolytic plaque assay. This assay is based on the ability of plasmin to degrade casein which gives rise to visible plaques on the agar background (Vassalli *et al.*, 1977). The murine LB6 cells produce no plasminogen activator (our unpublished observation), so plasmin cannot be produced. In the presence of uPA receptors, however, cells can bind uPA and hence acquire the ability to degrade casein in the presence of

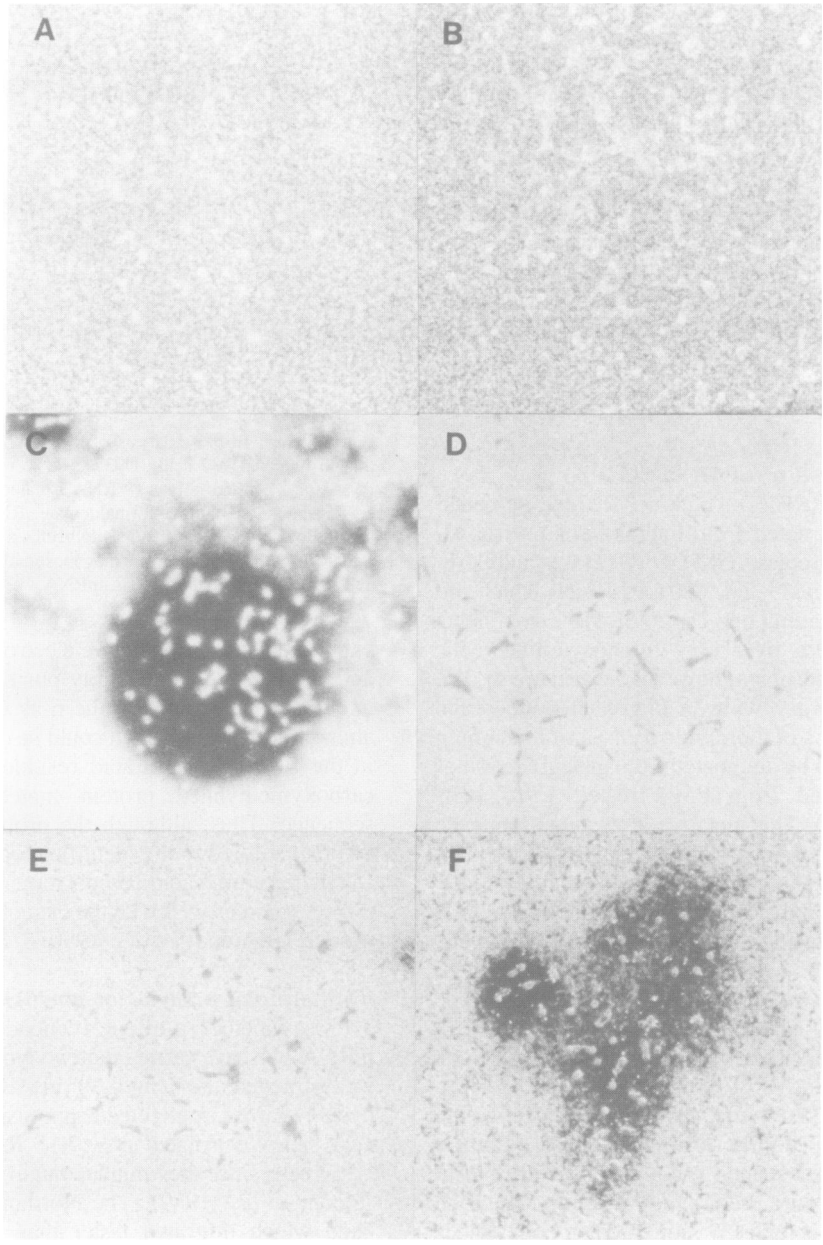

Fig. 3. Caseinolytic plaque assay of uPA binding to LB6 cells transfected with p-uPAR-1 DNA. (**A**) and (**C**–**F**) refer to clone LB6/p-uPAR-1, while (**B**) refers to clone LB6/RSVCAT. In (**A**) no uPA was added; otherwise (**B**–**F**) cells were subjected to a binding step with 0.2 nM human uPA for 1 h at 37°C. The following competitors, present during the binding step, were used: none (**B** and **C**); 100 nM ATF (**D**); 200 μM synthetic peptide human uPA(12-32[ala19]) (**E**); 100 μM synthetic peptide mouse uPA(13-33[ala20]) (**F**).

plasminogen (Vassalli *et al.*, 1985). Since binding is strictly species specific (D.Belin and J.-D.Vassalli, personal communication, 1985; Appella *et al.*, 1987; Estreicher *et al.*, 1989), LB6 cells do not bind human uPA and therefore they will score negative in a caseinolytic plaque assay, even after incubation with human uPA. Expression of human uPAR cDNA by LB6 cells, on the other hand, should allow them to bind human uPA and thus to form plaques. Purified p-uPAR-1 and p-RSV-neo DNA were co-transfected into mouse LB6 cells, a pool of G418-resistant clones was isolated and analyzed for human uPA binding (0.2 nM) by the caseinolytic plaque technique. Control experiments showed that all cells were negative in this assay in the absence of added uPA or plasminogen. After incubation with human uPA and in the presence of plasminogen the pool of G418-resistant cells that had received p-uPAR-1 DNA gave a high number of caseinolytic plaques; control cells (transfected with pRSVCAT and pRSVneo DNA) were negative (data not shown). Transfected cells were subcloned and single clones from each transfection expanded and retested for human uPA binding. The results obtained with one such clone at an early stage of cloning are shown in Figure 3. Most of the LB6 cells transfected with p-uPAR-1 DNA formed caseinolytic plaques upon binding human uPA (see panel C versus A), while those transfected with RSVCAT DNA did not (see panel B). Upon repeated cloning, all the cells became positive in this assay (data not shown). Specificity and saturability is shown by the ability of the truncated amino-terminal fragment (ATF) of uPA, and of the synthetic peptide human uPA[12-32(ala19)] to

compete with human uPA (Figure 3, panels D and E). On the contrary, the mouse peptide uPA[13-33(ala20)] does not compete for the binding (Figure 3F). These are the results predicted on the basis of the species specificity of uPA binding.

Expression of p-uPAR-1 DNA in LB6 cells is supported by quantitative binding data with ^{125}I-labeled ATF. Figure 4 shows a binding competition experiment where control LB6 cells (LB6/RSVCAT) did not bind ^{125}I-labeled ATF, while LB6 cells transfected with p-uPAR-1 DNA did. The binding was specifically competed by unlabeled ATF. A Scatchard plot of the data gave a K_d of ~10 nM and ~25 000 receptors/cell. In parallel experiments in which a human cell line (KB) was used as a source of uPAR, Scatchard plot analysis gave values of K_d ~1.0 nM (not shown).

In order to test whether the p-uPAR-1 expressed in the transfected LB6 cells has the correct molecular properties, we have carried out cross-linking studies with the LB6/p-uPAR-1 cells. Cells were incubated with human ^{125}I-labeled ATF, bound ATF was cross-linked with disuccinimidyl-suberate, and the cells were lysed and anlayzed by SDS–PAGE. The results are shown in Figure 5. Whereas the ligand ATF migrates with an M_r of ~17 000 daltons, the cross-linked ligand migrates with an M_r of ~67 000, as expected for the intact ATF–uPAR complex (Nielsen *et al.*, 1988). For comparison Figure 5 shows a cross-linking experiment in which human GM637 cells (from which the cDNA clone is derived) were used. Considering the possible cell and species dependent difference in glycosylation, the data obtained with the two cell types are in good agreement and correspond closely to those obtained with purified uPAR (Nielsen *et al.*, 1988; Behrendt *et al.*, 1990).

An alternative experiment was carried out to determine the subcellular location of the product of the transfected uPAR gene. Immunofluorescence analysis of the LB6/RSVCAT and LB6/p-uPAR-1 cells was performed, with and without preincubation (30 min at 37°C) with human uPA (10 nM). The bound protein was revealed with an anti-human uPA monoclonal antibody and a fluoresceinated anti-mouse IgG second antibody. While the LB6/RSVCAT cells are negative with or without preincubation with uPA, pre-incubation of human uPA with LB6/p-uPAR-1 cells makes them strongly positive. The fluorescence is clearly located at the cell membranes precisely following their contour (not shown).

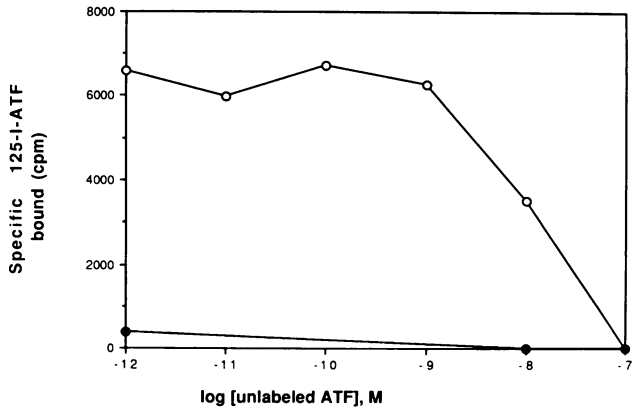

Fig. 4. Binding of human ^{125}I-labeled ATF to mouse LB6 cells transfected with p-RSVCAT (closed circles) and p-uPAR-1 DNA (clone 19) (open circles). Specific binding was calculated by subtracting the counts not competed by 100 nM unlabeled ATF (~1000 c.p.m. in this experiment).

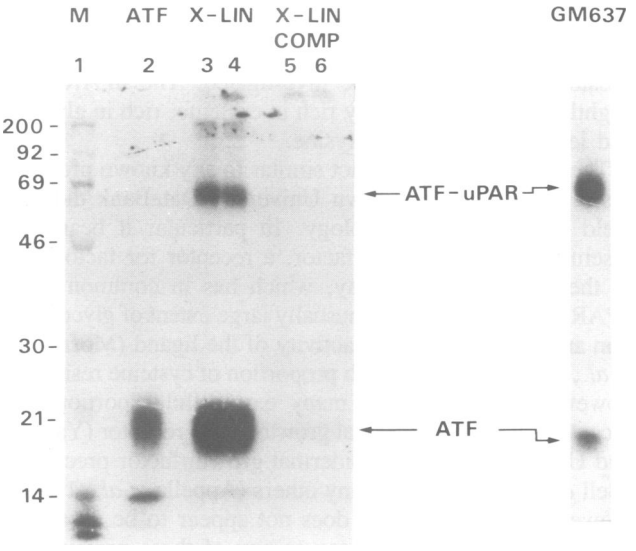

Fig. 5. Reducing SDS–polyacrylamide (12.5%) gel electrophoretic analysis of the ^{125}I-labeled ATF cross linked to LB6/p-uPAR-1 cells (clone 19). **Lane 1** has the M_r markers (see Materials and methods); **lane 2** represents the migration of labeled ATF (3000 c.p.m.); **lanes 3** and **4** show the migration of duplicate LB6-p-uPAR-1 extracts cross linked with ligand; **lanes 5** and **6** show the competition of cross linking of LB6/p-uPAR-1 cells to the ligand in the presence of 100 nM unlabeled ATF. The last lane to the right shows an example of cross linking obtained (in a separate experiment) with the same ligand and the human GM637 cells, which served as a source of RNA for the cDNA library used to isolate p-uPAR-1.

Discussion

Plasminogen dependent, and more specifically uPA dependent proteolysis is directly involved in cancer cell invasion and metastasis (Ossowski *et al.*, 1975; Reich, 1978; Ossowski and Reich, 1983; Danø *et al.*, 1985; Hearing *et al.*, 1988; Ossowski, 1988a,b; Axelrod *et al.*, 1989). This is based on the ability of plasmin to digest proteins of the extracellular matrix and the basement membrane which allows cells to migrate and invade (Bergman *et al.*, 1986; Mignatti *et al.*, 1986; Reich *et al.*, 1988). Surface uPA, i.e. bound to the receptor, appears to play a fundamental role in this process: in many cancer cells receptors are saturated with biosynthetic pro-uPA (Stoppelli *et al.*, 1986), which can be activated to two chain uPA by exogenous plasmin (Cubellis *et al.*, 1986) producing receptor bound active uPA (Cubellis *et al.*, 1989). The cellular location of biosynthetic uPA (cell to substratum and cell to cell contacts), suggests a direct role in cell shape and migration; at these sites uPA is bound to its receptor (Pöllanen *et al.*, 1987, 1988; Hebert and Baker, 1988; Stephens *et al.*, 1989).

The shape and migrating properties of normal and transformed cells can be modified in culture by a variety of agents, e.g. growth factors and phorbol esters (Ossowski *et al.*, 1975; Quigley, 1979). In RSV transformed chicken embryo fibroblasts these changes can be counteracted by anticatalytic anti-uPA antibodies (Sullivan and Quigley,

1986). Likewise, the properties of the uPAR are affected by these reagents. Treatment of U937 cells with PMA or with epidermal growth factor results in increased uPAR synthesis and decreased affinity for uPA (Stoppelli *et al.*, 1985; Nielsen *et al.*, 1988; Estreicher *et al.*, 1989; Picone *et al.*, 1989). The cloning and expression of the human uPAR molecule will facilitate studies aimed at understanding the role of the receptor in cell migration and invasiveness and the mechanisms of receptor regulation.

The human uPAR is a relatively small protein of 313 amino acid residues. The amino acid sequence contains five potential *N*-linked glycosylation sites, in agreement with the high level of glycosylation of the protein (Behrendt *et al.*, 1990). Starting at the amino acid position 282, a sequence of 21 hydrophobic amino acids, flanked by arginine residues, may represent the membrane spanning domain of the uPAR (Figure 1A). At the C-terminal (possibly intracellular) side of the presumptive membrane spanning segment, the arginine is followed by nine additional hydrophobic amino acids ending with a C-terminal threonine. Because of the high hydrophobicity of the 10 C-terminal residues, uPAR might contain no intracytoplasmic domain at all, i.e. the C-terminal 10 residues may also be buried in the membrane. This possibility will have to be investigated. The uPAR is a slightly acidic protein, very rich in cysteine, rich in glycine and leucine, and poor in lysine.

The uPAR sequence is not similar to any known protein: a search in the Georgetown University DataBank did not yield any extended homology. In particular it bears no resemblance to the tissue factor, a receptor for factor VII of the coagulation pathway, which has in common with uPAR a low mol. wt, an unusually large extent of glycosylation and a serine protease activity of the ligand (Morrissey *et al.*, 1987). The very high proportion of cysteine residues, however, is common to many extracellular portions of receptors, e.g. the epidermal growth factor receptor (Yarden and Ullrich, 1988), the epidermal growth factor precursor (Bell *et al.*, 1986), and many others (Appella *et al.*, 1988). However, in uPAR there does not appear to be a pattern of cysteine spacings common to any of these proteins.

Expression of human uPAR in mouse cells can be studied in view of the species specificity of binding. However, both mouse and human uPA bind their respective receptors through their growth factor domain, located at the N-terminus of the molecule (Appella *et al.*, 1987; Estreicher *et al.*, 1989). Expression of the human uPAR gene in mouse LB6 cells is demonstrated by the following findings: p-uPAR-1 DNA transfected LB6 cells bind labeled human ATF and unlabeled human uPA as shown by direct binding assay (Figure 4) and the caseinolytic plaque assay (Figure 3). The binding is specific as shown by the ability of human ATF and human synthetic peptide uPA[12-32(ala19)], but not mouse synthetic peptide uPA[13-33(ala20)], to compete for binding (Figures 3 and 4). The ATF–uPAR complex has the correct mol. wt (Figure 5) and the exogenously added human uPA can be located on the membrane of the LB6 cells in immunofluorescence studies (data not shown). However, the affinity for the ATF appears to be lower than that found in human cells (see Blasi, 1988). This result is very interesting as it might indicate that the affinity of the receptor for uPA is regulated by other proteins, and that these proteins are either absent in mouse LB6 cells or are also species specific as uPAR itself. Another possible explanation is that different, species specific, glycosylation is responsible for the different affinity. Appropriate experiments are required to clarify this point.

In conclusion, we have isolated, sequenced and functionally expressed the human uPAR cDNA. The availability of the uPAR cDNA will allow a detailed study of its interaction with the ligand, as well as of its role in the invasiveness and migration of malignant and normal cells.

Materials and methods

Cell culture and reagents

Mouse LB6 cells (Corsaro and Pearson, 1981) were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin.

Restriction enzymes, *Taq* polymerase, and other reagents for recombinant DNA were obtained from New England Biolabs, Boehringer Mannheim, NEN-Dupont Co and Pharmacia.

Human HMW urokinase and pro-urokinase were gifts from Lepetit SpA (Nolli *et al.*, 1989). The N-terminal fragment of human uPA, ATF, was a gift from Abbott Laboratories. Iodination of ATF has been described (Stoppelli *et al.*, 1985). The synthetic peptides, human uPA[12-32(ala19)] and mouse uPA[13-33(ala20)], have been described previously (Appella *et al.*, 1987). Plasminogen was from Sigma Chemical Co. The monoclonal antibody, clone 5, used in immunofluorescence experiments has been described (Nielsen *et al.*, 1986). The cross-linking reagent disuccinimidyl suberate was from Pierce Chemical Co.

Library screening and nucleotide sequencing

About 7×10^5 clones of the GM637 human fibroblasts cDNA library (Okayama and Berg, 1983) in HB101 cells were screened after chloramphenicol amplification (Maniatis *et al.*, 1982) using an oligonucleotide derived from a preliminary determination of the N-terminal sequence of human uPAR protein (Leu-X-X-Met-Gln-Asn-Lys-Thr-Asn-Gly-Asp) (data not shown). The sequence of the oligonucleotide is shown in panel B of Figure 1. This probe was end-labeled (Maniatis *et al.*, 1982) and hybridized to nitrocellulose filters (Schleicher & Schuell) in 5 × SSC at 50°C. Positive clones were purified and rescreened until pure. Three clones were sequenced using a synthetic oligonucleotide (5′ CAGTGGATGTTGCCTTTAC 3′) corresponding to the SV40 sequence present at the 5′ end of the insert (Okayama and Berg, 1983). The complete sequence of clone p-uPAR-1 DNA was obtained on double stranded DNA using both commercial M13 primers, used with p-EMBL-18 subcloned fragments, and internal synthetic primers which were used directly on the p-uPAR-1 DNA. The following internal oligonucleotides were used: TGAAGAACAGTGCCTGGA (nucleotides 466–483); CATCCAGGCACTGTTCTT (nucleotides 485–468); ATGGATGCTCCTCTGAAG (nucleotides 720–737); CCTGAGGTCACACAGCAA (nucleotides 1149–1132); CACAGTCTGGCAGTCATT (nucleotides 1029–1012) (see Figure 1A for location of the oligonucleotides).

RNA preparation and Northern blot analysis

U937 cells were treated with 100 ng/ml PMA for 24 h, the cells were harvested and poly(A)$^+$ RNA prepared as described elsewhere (Medcalf *et al.*, 1986). The RNA was electrophoresed through an agarose–formaldehyde gel (Lund *et al.*, 1987) and the migration of ribosomal RNAs measured from a control lane with total RNA. The RNA was transferred to a nitrocellulose filter (Schleicher & Schuell), and hybridized with nick-translated DNA probes. The entire p-uPAR-1 DNA was used as a probe for uPAR RNA. The blot was subsequently rehybridized with an actin probe (Ponte *et al.*, 1983), and the size of the RNA extrapolated on the basis of the migration of the three markers (28S, 18S and actin mRNA).

Transfection and caseinolytic plaque assay

LB6 cells (2×10^5) were transfected either with 9 μg p-uPAR-1 DNA plus 1 μg pRSVneo DNA, or with 9 μg pRSVCAT plus 1 μg pRSVneo DNA, using a modification of the calcium phosphate co-precipitation technique (Pozzatti *et al.*, 1986). Cells were plated in 0.8 mg/ml G418 containing DMEM with 10% fetal calf serum, and colonies were isolated after ~13 days. The pools of transfected clones were tested (in the case of p-uPAR-1 DNA) by the caseinolytic plaque assay (Vassalli *et al.*, 1977) and positive clones were picked. After one subcloning, several clones from each transfection were tested for human uPA binding, using the same technique. Cells (plated 1 day before at 100 000/dish), were washed with phosphate-

buffered saline (PBS), incubated in the presence of 0.2 nM human uPA for 1 h at 37°C, washed extensively and covered with a thin agar layer containing 1.3% casein, and 17 μg/ml plasminogen. The plates were incubated at 37°C for 3 h, stained with Coomassie brilliant blue R250 and photographed. In some experiments, specific competitors were used during the binding step (see Figure 3 legend for concentration).

Binding of ^{125}I-labeled ATF

About 300 000 LB6/RSVCAT or LB6/p-uPAR-1 (clone 19) cells in a 30 mm dish were washed with PBS containing 1 mg/ml bovine serum albumin (BSA) incubated in serum free medium for 1 h at 37°C, and then incubated with 47 000 c.p.m. ^{125}I-labeled ATF (1500 c.p.m./fmol) at 37°C for 60 min, in the presence of different concentrations of unlabeled ATF. The experiment was carried out in duplicate. At the end of the incubation the cells were washed with PBS/BSA, incubated for 15 min at 37°C in 0.5 N NaOH, and the cell lysate collected and counted (Stoppelli *et al.*, 1986). Specific binding was calculated by subtracting the radioactivity not competed by 100 nM ATF.

Cross-linking of ^{125}I-labeled ATF to the uPAR

Cross-linking of LB6/p-uPAR-1 cells with [^{125}I]ATF was carried out using disuccinimidyl suberate, as previously described (Picone *et al.*, 1989). Briefly, duplicate dishes of 2.6×10^5 cells were washed with PBS/BSA (1 mg/ml), incubated with 60 000 c.p.m. [^{125}I]ATF (1500 c.p.m./fmol) in serum-free DMEM supplemented with 25 mM HEPES, pH 7.4 for 60 min at 37°C, washed four times with PBS/BSA, and crosslinked with 1 mM DSS for 15 min at room temperature. Cross-linking was stopped with 10 mM (final concentration) ammonium acetate for 10 min at room temperature. Cells were scraped with PBS containing 1 mM EDTA, 1 mM PMSF, collected by centrifugation, resuspended in 25 μl distilled water, and counted. The cells were then lysed directly in Laemmli buffer containing 5% β-mercaptoethanol (Laemmli, 1970). In control samples, 100 nM unlabeled ATF was present during the binding step. The cell extract was analyzed by SDS–polyacrylamide (12.5%) gel electrophoresis under reducing conditions (Laemmli, 1970), along with mol. wt markers (myosin, phosphorylase b, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme) (Rainbow, Amersham). The gel was dried and exposed to X-ray film.

Immunofluorescence analysis

About 100 000 LB6/p-uPAR-1 cells were plated in DMEM 10% fetal calf serum on a glass cover slip in 60 mm dishes. After 24 h, cells were washed several times with PBS containing 1 mg/ml BSA, and then incubated in the presence of 10 nM uPA. Control samples were run in the absence of pro-uPA. After 30 min at 37°C, both control and uPA incubated cells were washed four times with PBS/BSA and incubated in the same medium in the presence of 30 μg/ml of anti-uPA monoclonal antibody (clone 5) (Nielsen *et al.*, 1986), for 20 min on ice. Controls without the anti-uPA antibody were carried out. Subsequently, cells were washed thoroughly and incubated for 20 min on ice with a 1:20 dilution of fluoresceinated anti-mouse IgG. Cells were observed and photographed in phase contrast and in fluorescence with a Zeiss Axiophot microscope.

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References

Appella,E., Robinson,E.A., Ulrich,S.J., Stoppelli,M.P., Corti,A., Cassani,G. and Blasi,F. (1987) *J. Biol. Chem.*, **262**, 4437–4440.

Appella,E., Weber,I.T. and Blasi,F. (1988) *FEBS Lett.*, **231**, 1–4.

Axelrod,J.H., Reich,R. and Miskin,R. (1989) *Mol. Cell. Biol.*, **9**, 2133–2141.

Bajpai,A. and Baker,J.B. (1985) *Biochem. Biophys. Res. Comm.*, **133**, 994–1000.

Behrendt,N., Rønne,E., Ploug,M., Petri,T., Løber,D., Nielsen,L.S., Schleuning,W.-D., Blasi,F., Appella,E. and Danø,K. (1990) *J. Biol. Chem.*, in press.

Bell,G.I., Fong,N.M., Stempien,M.M., Wormsted,M.A., Caput,D., Ku,I., Urdea,M.S., Rall,S.B. and Sanchez-Pescador,L. (1986) *Nucleic Acids Res.*, **14**, 8427–8446.

Bergman,B.L., Scott,R.W., Bajpai,A., Watts,S. and Baker,J.B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 996–1000.

Blasi,F. (1988) *Fibrinolysis*, **2**, 73–84.

Blasi,F., Vassalli,J.-D. and Danø,K. (1987) *J. Cell Biol.*, **104**, 801–804.

Corsaro,C.M. and Pearson,M.L. (1981) *Somat. Cell Genet.*, **7**, 603–616.

Cubellis,M.V., Nolli,M.L., Cassani,G. and Blasi,F. (1986) *J. Biol. Chem.*, **261**, 15819–15822.

Cubellis,M.V., Andreasen,P.A., Ragno,P., Mayer,M., Danø,K. and Blasi,F. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4828–4832.

Danø,K., Andreasen,P.A., Grøndahl-Hansen,J., Kristensen,P., Nielsen,L.S. and Skriver,L. (1985) *Adv. Cancer Res.*, **44**, 139–266.

Dente,L., Cesareni,G. and Cortese,R. (1983) *Nucleic Acids Res.*, **11**, 1645–1655.

Ellis,V., Scully,M.F. and Kakkar,V.V. (1989) *J. Biol. Chem.*, **264**, 2185–2188.

Estreicher,A., Wohlwend,A., Belin,D. and Vassalli,J.D. (1989) *J. Biol. Chem.*, **264**, 1180–1189.

Hearing,V.J., Law,L.W., Corti,A., Appella,E. and Blasi,F. (1988) *Cancer Res.*, **48**, 1270–1278.

Hebert,C. and Baker,J.B. (1988) *J. Cell Biol.*, **106**, 1241–1247.

Hopp,T.P. and Woods,K.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3824–3828.

Kozak,M. (1987) *Nucleic Acids Res.*, **15**, 8125–8132.

Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lund,L.R., Riccio,A., Andreasen,P.A., Nielsen,L.S., Kristensen,P., Laiho,M.,Saksela,O., Blasi,F. and Dano,K. (1987) *EMBO J.*, **6**, 1281–1286.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Medcalf,R.L., Richard,R.I., Crawford,R.J. and Hamilton,J.A. (1986) *EMBO J.*, **5**, 2217–2222.

Mignatti,P., Robbins,E. and Rifkin,D.B. (1986) *Cell*, **47**, 487–498.

Miles,L.A. and Plow,E.F. (1988) *Fibrinolysis*, **2**, 61–71.

Morrissey,J.H., Falhrai,H. and Edgington,T.S. (1987) *Cell*, **50**, 129–135.

Nielsen,L.A., Andreasen,P.A., Grøndahl-Hansen,J., Huang,J., Kristensen,P. and Danø,K. (1986) *Thromb. Haemostasis*, **55**, 206–212.

Nielsen,L.S., Kellerman,G.M., Behrendt,N., Picone,R., Danø,K. and Blasi,F. (1988) *J. Biol. Chem.*, **263**, 2358–2363.

Nolli,M.L., Sarubbi,E., Corti,A., Robbiati,F., Soffientini,A., Blasi,F., Parenti,F. and Cassani,G. (1989) *Fibrinolysis*, **3**, 101–106.

Okayama,H. and Berg,P. (1983) *Mol. Cell. Biol.*, **3**, 280–289.

Ossowski,L. (1988a) *Cell*, **52**, 312–328.

Ossowski,L. (1988b) *J. Cell Biol.*, **107**, 2437–2445.

Ossowski,L. and Reich,E. (1983) *Cell*, **35**, 611–619.

Ossowski,L., Quigley,J.P. and Reich,E. (1975) In Reich,E., Rifkin,D.B. and Shaw,E. (eds), *Proteases in Biological Control.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 901–913.

Picone,R., Kajtaniak,E., Nielsen,L.S., Behrendt,N., Mastronicola,M.R., Cubellis,M.V., Stoppelli,M.P., Danø,K. and Blasi,F. (1989) *J. Cell Biol.*, **108**, 693–702.

Pöllanen,J., Saksela,O., Salonen,E.-M., Andreasen,P., Nielsen,L., Danø,K. and Vaheri,A. (1987) *J. Cell Biol.*, **104**, 1085–1096.

Pöllanen,J., Hedman,K., Nielsen,L.S., Danø,K. and Vaheri,A. (1988) *J. Cell Biol.*, **106**, 87–95.

Ponte,P., Gunning,P., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 1783–1791.

Pozzatti,R., Muscel,R., Williams,S.J., Padmanabhan,R., Howard,B., Liotta,L. and Khoury,G. (1986) *Science*, **232**, 223–227.

Quigley,J.P. (1979) *Cell*, **17**, 131–141.

Reich,E. (1978) In Berlin,R.D., Herrmann,H., Lepow,I.H. and Tanzer,J.M. (eds), *Molecular Basis of Biological Degradative Processes.* Academic Press, New York, pp. 155–169.

Reich,R., Thompson,E.W., Iwamoto,Y., Martin,G.R., Deason,J.R., Fuller,G.C. and Miskin,R. (1988) *Cancer Res.*, **48**, 3307–3312.

Skriver,L., Larsson,L.-I., Kielberg,V., Nielsen,L.S., Andresen,P.B., Kristensen,P. and Danø,K. (1984) *J. Cell Biol.*, **99**, 753–758.

Stephens,R.W., Pöllanen,J., Tapiovaara,H., Leung,K.-C., Sim,P.-S.,

Salonen,E.M., Rønne,E., Behrendt,N., Danø,K. and Vaheri,A. (1989) *J. Cell Biol.*, **108**, 1987–1995.
Stoppelli,M.P., Corti,A., Soffientini,A., Cassani,G., Blasi,F. and Assoian,R.K. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4939–4943.
Stoppelli,M.P., Tacchetti,C., Cubellis,M.V., Corti,A., Hearing,V.J., Cassani,G., Appella,E. and Blasi,F. (1986) *Cell*, **45**, 657–684.
Sullivan,L.M. and Quigley,J.P. (1986) *Cell*, **45**, 905–915.
Vassalli,J.-D., Baccino,D. and Belin,D. (1985) *J. Cell Biol.*, **100**, 86–92.
Vassalli,J.-D., Hamilton,J. and Reich,E. (1977) *Cell*, **11**, 695–705.
von Heijne,G. (1986) *Nucleic Acids Res.*, **14**, 4683–4690.
Yarden,Y. and Ullrich,A. (1988) *Biochemistry*, **27**, 3113–3119.

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