u-PAR (urokinase-type plasminogen activator receptor), anchored to the cell surface via a glycolipid moiety, drives tumour progression. We previously reported that colon cancer cells (RKO clone 2, FS2), attenuated for in vivo tumorigenicity, are diminished >15-fold for u-PAR display when compared with their tumorigenic isogenic counterparts (RKO clone 2), this disparity not reflecting altered transcription/mRNA stability. FACS, confocal microscopy and Western blotting using a fused u-PAR–EGFP (enhanced green fluorescent protein) cDNA revealed a >14-fold differential in the u-PAR–EGFP signal between the isogenic cells, ruling out alternate splicing as a mechanism. Although metabolic labelling indicated similar synthesis rates, pulse–chase revealed accelerated u-PAR–EGFP turnover in the RKO clone 2 FS2 cells. Expression in RKO clone 2 cells of a u-PAR–EGFP protein unable to accept the glycolipid moiety yielded diminished protein amounts, thus mirroring the low endogenous protein levels evident with RKO clone 2 FS2 cells. Transcript levels for the phosphatidylglycan anchor biosynthesis class B gene required for glycolipid synthesis were reduced by 65% in RKO clone 2 FS2 cells, and forced overexpression in these cells partially restored endogenous u-PAR. Thus attenuated u-PAR levels probably reflects accelerated turnover triggered by inefficient addition of the glycolipid moiety.

Key words: urokinase-type plasminogen activator receptor (u-PAR), glycolipid anchor, protein degradation.

INTRODUCTION

u-PAR (urokinase-type plasminogen activator receptor), a 45–60 kDa glycosylated receptor [1] composed of three similar repeats [2], is connected to the cell surface via a GPI (glycosylphosphatidylinositol, i.e. a glycolipid) chain [3]. u-PAR is a multifunctional protein that plays a central role in proteolysis, cell migration and growth control and, as discovered more recently, in the suppression of fibrin-associated inflammation [4–9]. u-PAR contributes to these physiological functions via different mechanisms. First, the serine protease urokinase bound to this receptor activates plasminogen at a much faster rate than the fluid-phase plasminogen activator, thereby augmenting extracellular-matrix degradation. Secondly, u-PAR interacts with the extracellular domain of integrins, thereby mediating cell adhesion and migration [5] as evident in the re-epithelialization of skin wounds [10,11]. Thirdly, the u-PAR stimulates cell growth via both EGF (epidermal growth factor)-receptor-dependent and -independent signalling pathways [8,12]. Finally, it has been shown that the seven-transmembrane receptor FPR (formyl peptide receptor)-like receptor-1/lipoxin A4 receptor, a G-protein-coupled receptor, directly interacts with a soluble cleaved form of u-PAR to induce chemotaxis [13].

In cancer, there is strong evidence implicating u-PAR expression in tumour growth and progression. Indeed, elevated u-PAR levels are evident in various malignancies [14–16] and correlate with growth [8] and tumour progression [15]. Further, we previously reported that isogenic colon cancer cells (RKO clone 2 FS2) diminished 15-fold or more for cell-surface u-PAR display were attenuated for in vivo tumorigenicity when compared with their isogenic counterparts (RKO clone 2) enriched for this cell-surface receptor [17]. Finally, interfering with u-PAR expression or function retards the growth and invasiveness of some cancers, including glioblastomas [18–20].

u-PAR protein levels are controlled predominantly at the transcriptional level [21,22], although altered message stability [23,24] and translational efficiency [25] also contribute to the quantity of this gene product. Although we had experimentally ruled out a transcriptional role in diminished u-PAR display in the RKO clone 2 FS2 cells, characterized by their attenuated tumorigenicity [17], the post-transcriptional mechanism(s) responsible was not identified and is the focus of the present study. We report a novel mechanism by which u-PAR expression is controlled via an accelerated degradation of the protein secondary to interference with the addition of the glycolipid anchor to the protein.

EXPERIMENTAL

Cells, DNA constructs and antibodies

The colon cancer cell lines RKO clone 2 and its isogenic variant RKO clone 2 FS2 have been described previously [17]. Briefly, the RKO clone 2 cells were generated from the parental RKO colon cancer cell line by limiting dilution. The RKO clone 2 FS2 cells were subsequently derived from the RKO clone 2 cells by FACS for low endogenous u-PAR display.

The u-PAR–EGFP (enhanced green fluorescent protein) construct composed of the EGFP coding sequence inserted between the third domain of u-PAR and the GPI anchoring signal was as described elsewhere [26]. Glycosylation [27] and GPI-anchor mutations were as described previously [28], using
the u-PAR–EGFP construct as a template [26]. Mutations in u-PAR–EGFP were generated using the Stratagene QuikChange® II Site-Directed Mutagenesis kit. For glycosylation-site mutations, asparagine residues were replaced with glutamine residues. For the glycolipid-anchor mutations, the Stop 303 mutation was generated by inserting a stop codon (TGA) immediately after the alanine at position 303. The Thr Val Val u-PAR–EGFP plasmid was constructed by replacing the Ser-Gly-Ala residues (amino acids 282–284) with Thr-Val-Val. All u-PAR amino acid positions are as per EMBL accession number X51675. The Golgi- and ER (endoplasmic reticulum)-targeting constructs [Golgi–DsRed (Discosoma sp. red fluorescent protein) and ER–DsRed (both from Clontech)] encoded either the nucleotide sequences corresponding to the N-terminal 81 amino acids of human β-1,4-galactosyltransferase or the ER-targeting sequence of calreticulin fused to DsRed respectively.

Confocal microscopy

Cells were seeded in four-well chambers (Lab-Tek®; Nunc) and transfected using Lipofectamine™ 2000 (Invitrogen) on the following day with 0.5 μg of u-PAR–EGFP (wild-type or mutated in the u-PAR sequence) and 0.5 μg of a construct encoding ER/Golgi-targeting marker-protein sequences fused to DsRed (Clontech). On the following day, cells were washed with PBS, immediately after the alanine at position 303. The Thr Val Val u-PAR–EGFP plasmid was constructed by replacing the Ser-Gly-Ala residues (amino acids 282–284) with Thr-Val-Val. All u-PAR amino acid positions are as per EMBL accession number X51675. The Golgi- and ER (endoplasmic reticulum)-targeting constructs [Golgi–DsRed (Discosoma sp. red fluorescent protein) and ER–DsRed (both from Clontech)] encoded either the nucleotide sequences corresponding to the N-terminal 81 amino acids of human β-1,4-galactosyltransferase or the ER-targeting sequence of calreticulin fused to DsRed respectively.

Expression profiling

The Agilent human GE 4×44K microarray chip, which interrogates the entire expressed genome, was used. Triplicate sets of RNA were analysed and only those hits that were changed 2-fold or more in all three experiments and with a P value of <0.0001 were pursued for QPCR (quantitative PCR) validation using TaqMan® assays.

FACS

Transfected cells were harvested with 3 mM EDTA, washed with PBS and subjected to FACS analysis as described previously [17]. Transfection efficiencies were determined by co-transfection with either the Golgi–DsRed or ER–DsRed constructs. Transfection efficiency was quantified as the product of the percentage positive cells and mean fluorescence of the PE (phycoerythrin) channel.

QPCR

Total RNA (2 μg) was reverse transcribed and cDNA assayed by QPCR using the TaqMan® Gene Expression assay (Applied Biosystems). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used for normalization. Separate controls consisted of either deletion of the reverse transcriptase step or replacement of the cDNA mixture with distilled water.

Metabolic labelling and pulse–chase studies

Cells were washed with methionine/cysteine-free Eagle’s medium (Invitrogen) and incubated in methionine/cysteine-free medium [lacking FBS (fetal bovine serum)] for 15 min at 37°C. After this time, the cells were incubated with methionine/cysteine-free medium supplemented with Trans-label® (MP Biochemicals) (~0.2 mCi/ml final concentration; 1 Ci = 3.7×10¹⁰ Bq) for 30 min at 37°C. Cells were then washed with ice-cold PBS and extracted into a pH 7.4 buffer (10 mM Tris/HCl, 150 mM NaCl, 1.0% Triton X-100, 0.5% Nonidet P40, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 0.01 mg/ml aprotinin), and protein was immunoprecipitated with an anti-EGFP antisera (Clontech) diluted 1:500 (or with normal rabbit antiserum) and Protein A–agarose beads overnight. The immunoprecipitates were subjected to SDS/PAGE (10% gel), the gel was incubated with Amplify™ (Amersham) and radioactive protein was visualized by fluorography.

For chase experiments, after the 30-min labelling with Trans-label® at a final concentration of 0.2 mCi/ml, cells were washed with PBS, returned to 10% (v/v) FBS in McCoy’s 5A medium supplemented with 15 mg/l methionine for the designated times and harvested. Cell lysis and immunoprecipitations were carried out as described above.

Transfection

Transfections were performed using Lipofectamine™ 2000 according to the manufacturer’s instructions (Invitrogen) and as described previously [17].

Western blotting

Western blotting for PIG-B (phosphatidylglycan anchor biosynthesis class B) was with a 1:1500 dilution of an antibody generated against this protein (ProteinTech Group). Loading equality was checked by reprobing with an anti-β-actin antibody (1:1000 dilution). The positive control was lysate from human liver tissue. For the detection of endogenous u-PAR protein, Western blotting was performed using a rabbit anti-human u-PAR antibody (1:12000 dilution). Exogenous PIG-B expression was detected using an anti-GST (gluthathione transferase) antibody (Abcam) diluted to 1.25 μg/ml.

RESULTS

Differential expression of a u-PAR–EGFP-encoding construct in isogenic colon cancer cell populations

RKO clone 2 cells (generated from the parental RKO colon cancer cell line by limiting dilution) and RKO clone 2 FS2 cells derived by FACS of RKO clone 2 cells were used for low endogenous u-PAR display [17]. These isogenic colon cancer cells show a ~15-fold differential in u-PAR cell-surface display, a phenotype that could not be accounted for by changes in transcription or mRNA stability [17]. However, our investigations of the role of protein synthesis and/or turnover in the divergent u-PAR display were limited by the application of an anti-u-PAR antibody raised against the mature (heavily glycosylated) protein [1]. Thus, to overcome this drawback, we used an expression construct
Glycolipid-anchor-lacking urokinase receptor is degraded

Figure 1  Protein levels expressed from an exogenous u-PAR–EGFP construct mirrors endogenous u-PAR display

(A) The indicated cells were seeded in four-well chamber slides and on the following day transfected using Lipofectamine™ 2000 with the following DNA constructs: 0.5 μg of u-PAR–EGFP and 0.5 μg of a DsRed-tagged Golgi-targeting peptide. After 24 h, cells were fixed and imaged by confocal microscopy using the ×63 1.4 NA objective lens and oil immersion. Magnification is indicated by the 10-μm scale bar in each image. Arrows indicate Golgi bodies in the zoomed images. Images from independent fields (indicated as fields 1–4) and representative of triplicate experiments are shown for RKO clone 2 and RKO clone 2 FS2. (B) The indicated cells were transfected overnight with 1 μg each of the u-PAR–EGFP or ER–DsRed constructs or Lipofectamine™ only. Subsequently, cells were either analysed for transfection efficiency (C) as determined with ER–DsRed or were washed and replenished with serum-free medium. Conditioned medium and cell lysate were harvested 20 h later, the former concentrated and aliquots (50 μl) normalized for transfection efficiency were analysed by Western blotting using a 1:1500 dilution of the anti-EGFP antiserum.

encoding the EGFP coding sequence fused to u-PAR [26]. Since EGFP does not require post-translational modification [29], it allowed us to follow u-PAR biosynthesis/turnover without the protein-maturation variable confounding interpretation of the results.

Firstly, we determined whether the levels of the u-PAR–EGFP protein mirrored the disparate amount of endogenous u-PAR–EGFP protein in the RKO clone 2 (high) and RKO clone 2 FS2 (low) cells. Both cell populations were cotransfected with the u-PAR–EGFP DNA construct and a plasmid (ER–DsRed) encoding an ER-targeting sequence fused to the DsRed fluorescent protein, the latter enabling determination of transfection efficiencies. By FACS analysis, RKO clone 2 cells, enriched for cell-surface u-PAR [17], transfected with the u-PAR–EGFP construct showed a higher EGFP signal [mean EGFP: 18.7 and 6.0 respectively relative intensities] and a greater percentage of EGFP-positive cells (58.2 and 11.0 % respectively) when compared with similarly transfected RKO clone 2 FS2 cells (Supplementary Figure S1A at http://www.BiochemJ.org/bj/434/bj4340233add.htm), the latter displaying ~15-fold fewer surface u-PAR molecules [17]. For duplicate experiments, we calculated the expression of u-PAR–EGFP as the product of these two values normalized for transfection efficiency (Supplementary Figure S1B) and determined that RKO clone 2 cells were expressing 10–14-fold more u-PAR–EGFP protein compared with their isogenic counterparts (RKO clone 2 FS2), a difference that was quantitatively similar to that of the endogenous protein [17]. These results suggest that biosynthesis/turnover of the endogenous and u-PAR–EGFP-encoded proteins are very similar.
To further corroborate these findings, we transfected both endogenous u-PAR-rich (RKO clone 2) and u-PAR-poor (RKO clone 2 FS2) cells with the u-PAR–EGFP construct and performed confocal microscopy. Although strong EGFP positivity was evident with the RKO clone 2 cells (Figure 1A), the signal was barely detectable in the RKO clone 2 FS2 cells. This disparity could not be accounted for by altered transfection efficiency, assessed using a construct encoding a Golgi-targeting protein sequence fused to DsRed (Golgi–DsRed). Normalizing for transfection efficiency in five independent experiments, the EGFP signal in RKO clone 2 cells was 45-fold higher than in the isogenic RKO clone 2 FS2 cells as determined using ImageJ software. Interestingly, although u-PAR–EGFP clearly co-localized (yellow colour) with the Golgi apparatus in the RKO clone 2 cells (arrows in the merged fields in Figure 1A, zoomed images), which are enriched for endogenous u-PAR, the Golgi bodies in the RKO clone 2 FS2 cells, characterized by their attenuated endogenous u-PAR, were practically devoid of the exogenous protein (Figure 1A, arrows in zoomed images).

To determine whether the lower amount of cellular u-PAR–EGFP evident with the receptor-sparse RKO clone 2 FS2 cells was due to increased secretion/shedding, we analysed the conditioned medium for the exogenously expressed protein. However, the amount of u-PAR–EGFP in conditioned medium from these cells was barely detectable (Figure 2A, lane 6), which were enriched for endogenous u-PAR, the Golgi bodies in the RKO clone 2 FS2 cells, characterized by their attenuated endogenous u-PAR, were practically devoid of the exogenous protein (Figure 1A, arrows in zoomed images).

Increased turnover of u-PAR–EGFP protein in RKO clone 2 FS2 cells characterized by their low endogenous u-PAR

We then performed metabolic labelling experiments to determine whether the disparity in u-PAR display between the two isogenic cell populations was a consequence of altered synthesis or turnover. First, RKO clone 2 and RKO clone 2 FS2 cells were co-transfected where indicated with the u-PAR–EGFP and the ER–DsRed constructs, metabolically labelled with [35S]methionine/cysteine on the following day and extracted. Interestingly, although u-PAR–EGFP clearly co-localized (yellow colour) with the Golgi apparatus in the RKO clone 2 cells (arrows in the merged fields in Figure 1A, zoomed images), which are enriched for endogenous u-PAR, the Golgi bodies in the RKO clone 2 FS2 cells, characterized by their receptor-dense cell surface. Transfection efficiencies were similar for both cell populations (Figure 1C). Thus it is unlikely that the low amount of cell-associated u-PAR in the RKO clone 2 FS2 cells is due to a compensatory increase in shedding/secretion.

Abrogation of u-PAR protein glycosylation does not destabilize the u-PAR protein

We noted that the u-PAR–EGFP protein expressed in the RKO clone 2 FS2 cells, which are diminished for endogenous u-PAR, failed to show maturation (a broad diffuse band would indicate glycosylation [1]), as evidenced by a tight band in gel electrophoresis (Figure 2B). We thus entertained the possibility that aberrant glycosylation in these cells triggered its accelerated turnover, as occurs with some poorly glycosylated proteins [31,32]. To address this possibility, we replaced the u-PAR nucleotide sequences corresponding to the glycosylation sites [Asn31, Asn162 and Asn172 (construct D1–3), Asn200 and Asn303 (construct D2), or all five amino acids concurrently (construct UP5)] in the u-PAR–EGFP expression construct and transfected these plasmids into RKO clone 2 cells, which constitutively display a high cell-surface u-PAR number [17]. Interestingly, in comparison with the encoded wild-type protein, although the glycosylation-deficient proteins showed the expected lower molecular masses in Western blotting with little evidence (narrow rather than diffuse band) of maturation (Figure 3) in the RKO clone 2 cells, the intensities of the bands were comparable. As a control, expression of the wild-type construct in the RKO clone 2 FS2 cells, diminished for endogenous u-PAR, yielded almost undetectable levels of protein. Thus replacement of some, or all, of the u-PAR glycosylation sites in the exogenous construct did not accelerate turnover of the encoded protein. Furthermore, Affymetrix expression profiling of 1275 enzymes involved in deglycosylation/glycosylation using the GlycoV4 chip showed no difference in their expression between the RKO clone 2 and RKO clone 2 FS2 cells (results not shown).

Disruption of glycolipid anchoring accelerates u-PAR turnover in RKO clone 2 cells

In pulse-labelling experiments, we observed a consistent increase in the molecular mass of u-PAR–EGFP at zero time (Figure 2B, compare lane 4 with lane 3) for the RKO clone 2 FS2 cells in comparison with RKO clone 2 cells. Removal of the C-terminal 30 amino acids is a prerequisite for addition of the glycolipid
Glycolipid-anchor-lacking urokinase receptor is degraded

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Figure 2  u-PAR–EGFP protein turnover is increased in RKO clone 2 FS2 cells attenuated for endogenous u-PAR display

(A) The indicated cells in 100-mm-diameter dishes were transfected where indicated with u-PAR–EGFP/ER–DsRed (10 μg and 2.5 μg of DNA respectively) using Lipofectamine™ 2000 or, as a control, Lipofectamine™ 2000 only. Transfection efficiency determined by FACS (the product of the percentage of DsRed-positive cells and the mean fluorescence in the PE channel) was within 25% for the two cell populations. After ~20 h, the cells were pulse-labelled with [35S]Trans-label® (methionine/cysteine) for 30 min, and the indicated lysate protein amount was immunoprecipitated overnight using a 1:500 dilution of anti-EGFP antiserum or an equivalent amount of normal IgG. Immunoprecipitated proteins were resolved by SDS/PAGE and visualized by fluorography. (B) These were carried out as described in (A) with the exception that following the pulse, cells were supplemented with an excess of non-radioactive methionine, changed to 10% (v/v) FBS/McCoy’s 5A medium and returned to the incubator. At the indicated chase times, cell lysate was either subjected to Western blotting with an anti-β-actin antibody (1:1000) or immunoprecipitated (500 μg of cell lysate protein) with the anti-EGFP antiserum as described for (A). Results are shown for one of two experiments. The upper and lower panels represent different film exposure times. (C) Densitometry of the u-PAR–EGFP signal in the chase experiments. Note that the composite data include additional time points (2 h and 6 h) used in one of the two chase experiments. Results are means ± range; error bars indicate data ranges corresponding to those time points common to both experiments. Black circles, RKO clone 2 cells; white circles, RKO clone 2 FS2 cells.

anchor [33,34] and therefore we hypothesized that interference with the addition of the glycolipid anchor could destabilize the u-PAR protein. To answer this question, we generated constructs in which (i) the u-PAR–EGFP was replaced at Ser282-Gly283-Ala284 (construct ThrV alV al), corresponding to the target residues to which the glycolipid anchor is added [28], and (ii) the last ten C-terminal u-PAR residues were deleted by the introduction of a stop codon (construct Stop 303). Although the glycolipid anchor is not conjugated to any of these ten residues, this decapeptide is required for optimal addition of the glycolipid moiety at residues 282–284 [28]. Interestingly, in Western blotting, expression of the u-PAR–EGFP ThrValVal construct (thus abrogating addition of the glycolipid anchor) in the RKO clone 2 cells yielded a substantially lower amount of protein when compared with the wild-type control (Figure 4A), similar to the diminished amount of endogenous u-PAR protein evident in RKO clone 2 FS2 cells (Figure 4B). Expression of the u-PAR–EGFP Stop 303 construct encoding a protein that retains the glycolipid-attachment residues (282–284) (but in which the terminal ten amino acids required for optimal linkage of this moiety were deleted) also gave a lower amount of protein compared with the wild-type control. The attenuated signal generated with the glycolipid mutants was not due to their diminished synthesis as determined in metabolic labelling experiments (Figure 4C). These results are consistent with the notion that interference with the addition of the GPI anchor to u-PAR accelerates degradation of this protein. Unfortunately, the extremely low steady-state levels of endogenous u-PAR in RKO clone 2 FS2 cells (Figure 4B and [17]) precluded a protein analysis to determine the glycolipid status of this protein.

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Asn162 and Asn172 (construct D1–3), at Asn200 and Asn303 (construct D2), or at all five asparagine residues concurrently (UP5). After 40–48 h, the cells were harvested and lysed and 20 μg of protein was subjected to Western blotting using a 1:1500 dilution of the anti-EGFP antiserum. The experiment was repeated twice.

Next, we undertook genome-wide expression profiling to query gene products with established roles in glycolipid anchoring [35–37] that were differentially expressed in the RKO clone 2 and RKO clone 2 FS2 cells. However, at a relatively modest cutoff of a 2-fold change, we could not identify any known genes encoding proteins that participate in the addition of the GPI anchor with one exception, the PIG-B transcript whose expression was reduced in the RKO clone 2 FS2 cells characterized by their low endogenous u-PAR levels (Supplementary Table S1 at http://www.BiochemJ.org/bj/434/bj4340233add.htm). QPCR for PIG-B in four independent assays confirmed a 65% reduction in the RKO clone 2 FS2 cells when the u-PAR sequence was either wild-type (wt) or mutated at Asn152, Asn154 and Asn157 (construct D1–3), at Asn200 and Asn303 (construct D2), or at all five asparagine residues concurrently (UPS). After 40–48 h, the cells were harvested and lysed and 20 μg of protein was subjected to Western blotting using a 1:1500 dilution of the anti-EGFP antiserum. The experiment was repeated twice.

We then entertained the notion that abortive u-PAR biosynthesis might result in an aberrant protein selected for ER-associated degradation [31] or turnover in the lysosomal compartment [38]. To answer this question, we employed inhibitors to these degradative pathways. First, RKO clone 2 FS2 cells were transfected with the u-PAR–EGFP-encoding plasmid and subsequently treated with either Bortazomib or lactacystin using concentrations of these agents that are known to block these degradative pathways [39,40]. However, by confocal microscopy we could see little evidence of restoration of the u-PAR–EGFP signal in the RKO clone 2 FS2 cells (Supplementary Figure S2A at http://www.BiochemJ.org/bj/434/bj4340233add.htm) with Bortazomib, although Western blotting indicated induction of p21 (Supplementary Figure S2B), a known proteasomal target [39,41]. Treatment with lactacystin [32] caused a partial rescue of u-PAR–EGFP protein levels, but not to the level evident with RKO clone 2 cells enriched for endogenous u-PAR (Figure 5A). The partial rescue may reflect in part the post-transfection addition of the agents. Nevertheless, it is likely that the proteasomal pathway at best has a minor role in u-PAR turnover subsequent to aberrant biosynthesis. Similar experiments using the lysosomotropic alkaloid agent chloroquine [4] failed to rescue u-PAR–EGFP protein levels in RKO clone 2 FS2 cells (Figure 5B), arguing against a role for this pathway in degrading this protein.

**DISCUSSION**

We had previously reported a 15-fold reduction in u-PAR display in a subpopulation of RKO clone 2 colon cancer cells, a property that correlated with their diminished in vivo tumorigenicity [17]. However, attempts to identify the mechanism underlying this differential in u-PAR display were limited by the reagents available at the time. Using an expression construct encoding the u-PAR coding sequence fused to an EGFP tag, the present study strongly suggests that attenuated endogenous u-PAR levels in the RKO FS2 cells is a consequence of the accelerated turnover of this protein. Moreover, the trigger for accelerated u-PAR degradation is the absence of the glycolipid anchor from the protein. To our knowledge, this is the first study to show that inefficient addition of the glycolipid moiety leads to increased catabolism of the u-PAR protein.

Our studies clearly ruled out various alternative mechanisms that could potentially account for the divergence in cell-surface u-PAR display in these isogenic colon cancer cells. As an example, in using a cDNA encoding the u-PAR protein sequence, we could eliminate alternative splicing of the transcript [42] as a contributing factor to the differential in display. A second possibility was that decreased protein synthesis was the underlying cause for the reduction in u-PAR display in the RKO clone 2 FS2 cells, as reported previously in human myeloid leukocytes [25]. However, our metabolic labelling studies performed in conjunction with an antibody directed at EGFP showed no difference in u-PAR synthesis between the cells
Glycolipid-anchor-lacking urokinase receptor is degraded

Figure 4 Interference with the addition of the glycolipid moiety to the u-PAR–EGFP protein promotes u-PAR protein instability

RKO clone 2 cells were transfected with no DNA or 12.5 μg of the indicated constructs, ThrValVal (substitutions at u-PAR amino acids 282–248) and Stop 303 (in which a stop codon was introduced at codon 304 in the u-PAR protein sequence). Cells were harvested at 24 h post-transfection and analysed either by Western blotting (A) using a 1:1500 dilution of the anti-EGFP antiserum, or metabolically labelled for 30 min and 500 μg of protein immunoprecipitated with the anti-EGFP antiserum as described in Figure 2(A) and subjected to fluorography (C). Results are typical of replicate experiments. (B) Western blotting of endogenous u-PAR using a polyclonal antibody. (D) Western blotting of cell lysates from the indicated cells using an anti-PIG-B antibody. The positive control was a lysate of human liver. (E) RKO clone 2 FS2 cells were transfected with an empty vector or 20 μg of the PIGB–GST expression construct. Cells were harvested ∼38 h later and Western blotted for endogenous u-PAR protein, exogenous PIG-B (using an anti-GST antibody) and actin as a loading control.

characterized by their high (RKO clone 2) and low (RKO clone 2 FS2) cell-surface display of endogenous u-PAR. An alternative hypothesis was that inefficient u-PAR glycosylation provided a stimulus for protein turnover via ER-associated degradation in which unfolded or malfolded proteins are retained in the ER and then retro-translocated to the cytoplasm to be degraded [31]. Indeed, the lack of u-PAR protein maturation in RKO clone 2 FS2 (diminished for endogenous u-PAR), but not with the RKO clone 2 cells (enriched in u-PAR display), in pulse–chase studies was consistent with this notion. However, other results made this contention less probable. First and foremost, transfection of u-PAR–EGFP constructs mutated at the five potential glycosylation sites [27] in the RKO clone 2 cells enriched for endogenous u-PAR, but not with the RKO clone 2 cells (enriched in u-PAR display), in pulse–chase studies was consistent with this notion. However, other results made this contention less probable. First and foremost, transfection of u-PAR–EGFP constructs mutated at the five potential glycosylation sites [27] in the RKO clone 2 cells enriched for endogenous u-PAR gave rise to protein products which, although showing little evidence of maturation, nevertheless were similar in amount compared with the exogenous wild-type protein. Secondly, genes (Bip, GRP94, DDIT3 and Gadd153) known to be transcriptionally up-regulated [43] in the unfolded protein response were unchanged in RKO clone 2 FS2 cells as determined by expression profiling.

On the other hand, our pulse–chase experiments clearly indicated increased u-PAR catabolism in the RKO clone 2 FS2 cells attenuated in their display of the cell-surface protein. Accelerated u-PAR turnover has been reported previously [44], with VLDLR (very-low-density lipoprotein receptor) triggering this catabolism through internalization of urokinase–PAI-1 (plasminogen activator inhibitor-1). However, again our expression profiling indicated no change in the level of expression of VLDLR or PAI-1, making it unlikely that the trigger for increased catabolism in RKO clone 2 FS2 cells was that reported for MDA-MB-435 mammary adenocarcinoma cells [44].

So, what might lead to accelerated u-PAR turnover in the colon cancer cells attenuated in endogenous u-PAR? In metabolic labelling experiments, we consistently noticed that the molecular size of newly synthesized u-PAR (zero time, Figure 2B) for the RKO clone 2 FS2 cells, which display 15-fold fewer u-PAR molecules, was higher than the corresponding band with the u-PAR protein-rich RKO clone 2 cells. Typically, when the GPI moiety is added to newly synthesized u-PAR in the ER [33], the preceding step involves the removal of the last 30
C-terminal amino acids [28]. Omission of this cleavage could account for the initial increase in molecular mass evident with u-PAR–EGFP synthesized in the RKO clone 2 FS2 cells. In view of these results, we predicted that if interfering with the addition of the glycolipid moiety to u-PAR triggered its increased catabolism, then expression of a u-PAR–EGFP construct in which the residues at positions 282–284 are replaced to abrogate addition of the glycolipid anchor in the RKO clone 2 cells (enriched for endogenous u-PAR) should yield a low u-PAR–EGFP level akin to that evident for endogenous u-PAR in the RKO clone 2 FS2 cells. Indeed, this proved evident, arguing strongly that u-PAR protein deficient in the glycolipid anchor triggers accelerated degradation. This contention is consistent with a prior study in which the replacement of the identical u-PAR residues (282–284) to ablate the addition of the glycolipid moiety yielded a reduced total amount of u-PAR, an observation that was not commented upon in the original publication [28].

Although secretion of u-PAR, as evident with leucocytes from patients with paroxysmal nocturnal haemoglobinuria [45], could theoretically account for the lower amount of cellular protein in the RKO clone 2 FS2 cells, several observations failed to support such a mechanism. First, the amount of u-PAR–EGFP in the conditioned medium from the RKO clone 2 FS2 cells was barely detectable, in contrast with the strong signal evident with the RKO clone 2 cells. Secondly, if u-PAR secretion was evident in the RKO clone 2 FS2 cells, then transit of the EGFP-fused protein through the Golgi apparatus should be readily detectable by confocal microscopy in these cells. This was not the case, although u-PAR–EGFP consistently co-localized with these organelles in RKO clone 2 cells enriched for endogenous u-PAR. Thirdly, it is difficult to conceptualize how the near absence of cell-associated u-PAR, as determined by Western blotting of RKO clone 2 FS2 cells, could be completely accounted for by re-routing of this protein to the extracellular compartment. Interestingly, previous reports have indicated that the presence of the glycolipid moiety fused to target proteins is indispensable for ER exit [35] and that proteins lacking this moiety are thereafter degraded [35].

If in fact accelerated u-PAR turnover reflects deficient machinery with regard to the addition of the GPI moiety, where might such a deficiency reside? Our expression profiling to interrogate the entire human genome for genes {e.g. various PIGα and MPPE1 (metallophosphoesterase 1) [35]} known to participate in the addition of the GPI moiety to proteins [36] failed to identify any genes that were altered in their expression by 30% or more between the isogenic cells, with one exception. In expression-profiling experiments corroborated by QPCR and Western blotting, PIBB, encoding an α-1,2-mannosyltransferase involved in one of the mannosylation steps, was decreased 65±7% in the RKO clone 2 FS2 cells diminished for endogenous u-PAR. It may be that attenuated expression of this enzyme yields a u-PAR species lacking the GPI anchor and thus is targeted for degradation. However, an alternative possibility is that a gene encoding one of the enzymes involved in the addition of the glycolipid anchor to u-PAR is mutated in the RKO clone 2 FS2 cells. Indeed, in paroxysmal nocturnal haemoglobinuria, PIGA is somatically mutated [46], and in inherited GPI deficiency, the promoter of the PIGM gene is substituted to decrease its expression by 99% [46]. Although expression profiling would clearly reveal the effect of the latter, a mutation that alters enzyme activity would not be detected. Nevertheless, we think it unlikely that somatic mutations in PIGA or, for that matter, any of the genes involved in biosynthesis and/or addition of the glycolipid moiety to u-PAR is the root cause of the divergence in u-PAR display in our isogenic cells. Thus, as we reported previously [17], the u-PAR-deficient RKO clone 2 FS2 cells spontaneously revert over time to high u-PAR display, and it is difficult to conceive how a DNA mutation would be re-substituted to the wild-type nucleotide sequence. Perhaps also germane to our study is that in Burkitt’s lymphoma, silencing of PIGL and PIGY via reversible promoter methylation generates GPI-anchor protein-deficient cell populations [47]. Additionally, and of particular interest, is that cell populations oscillating between high and low membrane GPI protein were also evident with the Burkitt’s lymphoma cell lines [47], reminiscent of our previous study with u-PAR display [17].

Figure 5  Proteasomal/lysosomal inhibitors only partially restore u-PAR–EGFP protein levels in RKO clone 2 FS2 cells

The indicated cells were transfected with u-PAR–EGFP and ER–DsRed as described in the legend to Figure 1 and thereafter cells were treated with the indicated concentration of Lactacystin or chloroquine for the specified times. Subsequently, the cells were processed for u-PAR–EGFP expression (A and B) as described for Figure 3.
It is interesting to speculate that addition of the glycolipid anchor to the u-PAR occurs prior to glycosylation of the protein and may even be a prerequisite for the latter modification. Thus we saw very little evidence of u-PAR glycosylation in the RKO clone 2 FS2 cells in the pulse–chase experiments, whereas a diffuse band (indicative of protein maturation) continued to completion in the Golgi apparatus [48]. Since addition of the glycolipid moiety to target proteins is necessary for efficient transport from the ER [35], it may be that little newly synthesized u-PAR arrives at the Golgi for completion of glycosylation.

In conclusion, we have described a novel mechanism of regulating u-PAR expression whereby interference with the addition of the glycolipid anchor to the protein triggers its accelerated catabolism. This mechanism is distinct from the more common secretion/shedding of glycolipid anchor-deficient u-PAR, as occurs in paroxysmal nocturnal haemoglobinuria [45].

REFERENCES

FUNDING

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTION

Hector Avila performed Western blotting and FACS analysis, metabolic labelling and expression profiling. Heng Wang and Sean Hartig participated in confocal microscopy. Santosh Chauhan performed PIG-B re-expression and Western blotting. Sean Hartig participated in the quantification and interpretation of these data. Douglass Boyd initiated the project and contributed to experimental design, data analysis and preparation of the paper.

We thank Dr Nicoliai Sidenius (San Raffaele Scientific Institute, Milan, Italy) and Dr Kang Zhang (Munir Eye Center, University of Utah, Salt Lake City, UT, U.S.A.) for the gifts of the u-PAR–EGFP and pDsRed2–ER constructs respectively. The pME–GST human PIG-B DHA neo plasmid was provided by Dr Noriyuki Kanazawa (Department of Immunoregulation, Osaka University, Osaka, Japan). We express our gratitude to Dr Steven Head (Scripps Research Institute, La Jolla, CA, U.S.A.) for expression profiling of glycosylation enzymes and to Dr Zhengxin Wang for intellectual input. The anti-u-PAR antibody was a gift from Dr Andrew Mazar (Angstrom Pharmaceuticals, San Diego, CA, U.S.A.). Mutations of the u-PAR–EGFP constructs were kindly generated by Hua Wang (MD Anderson Cancer Center).

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It is interesting to speculate that addition of the glycolipid anchor to the u-PAR occurs prior to glycosylation of the protein and may even be a prerequisite for the latter modification. Thus we saw very little evidence of u-PAR glycosylation in the RKO clone 2 FS2 cells in the pulse–chase experiments, whereas a diffuse band (indicative of protein maturation) became clearly evident over time with the RKO clone 2 cells. Similarly, in Western blotting, expression of the u-PAR–EGFP construct mutated to attenuate, or eliminate, addition of the glycolipid anchor again yielded proteins that showed minimal band heterogeneity in Western blots. Synthesis and addition of the glycolipid to target proteins is known to occur in the ER [33], but glycosylation (including N-glycosylation for proteins such as u-PAR [1]), which also starts in this subcellular structure, continues to completion in the Golgi apparatus [48]. Since addition of the glycolipid moiety to target proteins is necessary for efficient transport from the ER [35], it may be that little newly synthesized u-PAR arrives at the Golgi for completion of glycosylation.

In conclusion, we have described a novel mechanism of regulating u-PAR expression whereby interference with the addition of the glycolipid anchor to the protein triggers its accelerated catabolism. This mechanism is distinct from the more common secretion/shedding of glycolipid anchor-deficient u-PAR, as occurs in paroxysmal nocturnal haemoglobinuria [45].


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SUPPLEMENTARY ONLINE DATA

Accelerated urokinase-receptor protein turnover triggered by interference with the addition of the glycolipid anchor

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See the pages that follow for Supplementary Figures S1 and S2, and Supplementary Table S1.

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**Figure S1** u-PAR–EGFP protein is differentially displayed on RKO clone 2 and RKO clone 2 FS2 cells

The indicated cells were transfected with 1 μg each of the specified expression constructs or the two combined. pcDNA3.1 was used for back-filling to a constant DNA input. After 21 h, the cells were EDTA-harvested and subjected to FACS (A). FITC and PE channels correspond to u-PAR–EGFP and ER–DsRed respectively. To quantitate the u-PAR–EGFP signal, the percentage of EGFP-positive cells was multiplied by the mean fluorescence value in the FITC channel. Transfection efficiency was the product of the percentage of positive DsRed cells and the mean fluorescence value in the PE channel. Normalized u-PAR–EGFP expression was derived by dividing the u-PAR signal by the corresponding transfection efficiency value and multiplying by 100. The table (B) shows results from two independent experiments.
Figure S2  Bortazamib does not restore u-PAR–EGFP display

The indicated cells were transfected with u-PAR–EGFP and ER–DsRed as described in the legend to Figure 1 in the main paper and cells were thereafter treated with the indicated concentration of Bortazamib for the specified times. Subsequently, the cells were processed for u-PAR–EGFP expression by confocal microscopy as described in Figure 1(A) in the main paper or Western blotting for p21.
Table S1  Differentially expressed genes in the RKO clone 2 and RKO clone 2 FS2 populations

The Agilent human GE 4×44K microarray chip (G4112F; Agilent), which interrogates the entire expressed genome, was used. Triplicate sets of RNA were analysed and only those hits that were changed 2-fold, or more, in all three experiments and at a P value of <0.0001 were pursued for QPCR validation using TaqMan® assays. NLS, nuclear localization sequence.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change</th>
<th>P value</th>
<th>Description</th>
<th>Validated by QPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Repressed genes in RKO clone 2 FS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AY102069</td>
<td>22.9</td>
<td>6.14568 × 10−21</td>
<td>Homo sapiens surfactant associated protein F mRNA, partial sequence [AY102069] (pseudogene)</td>
<td>Not done</td>
</tr>
<tr>
<td>ICAM2</td>
<td>22.7</td>
<td>3.04887 × 10−21</td>
<td>Type I transmembrane glycoprotein; binds leucocyte adhesion LFA-1 protein; possible role in lymphocyte recirculation</td>
<td>Not done</td>
</tr>
<tr>
<td>IF27</td>
<td>11.5</td>
<td>1.967 × 10−19</td>
<td>119 amino acids, multi-pass membrane protein; up-regulated in skin and certain epithelial cancers</td>
<td>Not done</td>
</tr>
<tr>
<td>IF16</td>
<td>5.9</td>
<td>2.52353 × 10−16</td>
<td>H. sapiens interferon γ-inducible protein 16 (IFI16), mRNA [NM_005531]</td>
<td>Yes</td>
</tr>
<tr>
<td>RAC2</td>
<td>3.1</td>
<td>1.68346 × 10−10</td>
<td>H. sapiens ras-related C3 botulinum toxin substrate 2 (rho family, small GTP-binding protein Rac2) (RAC2), mRNA [NM_002872]</td>
<td>Yes</td>
</tr>
<tr>
<td>PIGB</td>
<td>2.2</td>
<td>7.34166 × 10−5</td>
<td>H. sapiens PIG-B, mRNA [NM_004855]</td>
<td>Yes</td>
</tr>
<tr>
<td>(b) Induced genes in RKO clone 2 FS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMP1</td>
<td>3.1</td>
<td>1.05314 × 10−10</td>
<td>H. sapiens epithelial membrane protein 1 (EMP1), mRNA [NM_001423]</td>
<td>Yes</td>
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<tr>
<td>TMSB4X</td>
<td>3.9</td>
<td>1.94845 × 10−12</td>
<td>H. sapiens thymosin β4, X-linked (TMSB4X), mRNA [NM_021109]</td>
<td>Not done</td>
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<tr>
<td>CDK2A</td>
<td>3.4</td>
<td>1.56128 × 10−11</td>
<td>Cyclin-dependent kinase inhibitor 2B (induces cell-cycle arrest in G1/G2, acts as tumour suppressor)</td>
<td>Not done</td>
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<tr>
<td>BNC2</td>
<td>2.9</td>
<td>4.28919 × 10−10</td>
<td>H. sapiens basonuclin 2 (BNC2), mRNA [NM_017637]; has NLS and three paired zinc fingers, may be transcription factor</td>
<td>Not done</td>
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<tr>
<td>TMSL3</td>
<td>3.4</td>
<td>1.2593 × 10−11</td>
<td>Thymosin-like 5; role in organization of cytoskeleton; binds and sequesters actin monomers</td>
<td>Not done</td>
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<tr>
<td>ENST00000334647</td>
<td>3.0</td>
<td>1.84219 × 10−10</td>
<td>H. sapiens phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homologue, Drosophila) (PDE4B), transcript variant d, mRNA [NM_011037341]</td>
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<tr>
<td>PDE4B</td>
<td>7.3</td>
<td>2.40427 × 10−5</td>
<td>H. sapiens phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homologue, Drosophila) (PDE4B), transcript variant d, mRNA [NM_011037341]</td>
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</tr>
<tr>
<td>CACNA2D1</td>
<td>6.2</td>
<td>8.24002 × 10−13</td>
<td>Voltage-gated calcium channel subunit α2</td>
<td>Not done</td>
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<tr>
<td>RASSF8</td>
<td>24.0</td>
<td>3.28418 × 10−17</td>
<td>419 amino acids; 48kDa; Ras association domain family 8</td>
<td>Yes</td>
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<tr>
<td>ZNF426</td>
<td>27.3</td>
<td>8.75634 × 10−19</td>
<td>554 amino acids; involved in transcription?</td>
<td>Not done</td>
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<tr>
<td>SPP1 (transcript variant 2)</td>
<td>40.0</td>
<td>1.47076 × 10−21</td>
<td>Cytokine enhancing interferon-γ- and interleukin-12 and reducing IL-10 production;</td>
<td>Yes</td>
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<tr>
<td>GPC6</td>
<td>15.4</td>
<td>8.07033 × 10−19</td>
<td>GPI-anchored proteoglycans with possible role in growth and cell division; putative cell-surface co-receptor for growth factors, extracellular matrix proteins, proteases</td>
<td>Not done</td>
</tr>
<tr>
<td>MAGEB2</td>
<td>11.8</td>
<td>4.37712 × 10−18</td>
<td>xp22-p21 linked; expressed in testis and placenta and in tumours of various histological origins</td>
<td>Yes</td>
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<tr>
<td>RGS4</td>
<td>14.2</td>
<td>1.82556 × 10−20</td>
<td>Drive G-proteins into their inactive GDP-bound forms; chromosome 1q23.3</td>
<td>Yes</td>
</tr>
<tr>
<td>C1orf110</td>
<td>38.2</td>
<td>2.61556 × 10−20</td>
<td>Hypothetical protein 302 amino acids of 34 kDa, chromosome 1q23.3</td>
<td>Not done</td>
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</tbody>
</table>