Receptor-mediated endocytosis of urokinase-type plasminogen activator is regulated by cAMP-dependent protein kinase

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SUMMARY

Internalization of the urokinase-type plasminogen activator (uPA) requires two receptors, the uPA receptor (uPAR) and the low density lipoprotein receptor-related protein (LRP)/α2-macroglobulin (α2M) receptor. Here, we address whether protein kinases are involved in the internalization of uPA by human melanoma cells. Initially, we found that the internalization of uPA was significantly inhibited by the serine/threonine protein kinase inhibitors staurosporine, K-252a and H-89, but not by the tyrosine kinase inhibitors, genistein and lavendustin A. Internalization of uPA was also inhibited by a pseudosubstrate peptide for cAMP-dependent protein kinase (PKA), but not by a pseudosubstrate peptide for protein kinase C. We confirmed a requirement for PKA-activity and implicated a specific isoform by using an antisense oligonucleotide against the regulatory subunit RIα of PKA which suppresses PKA-I activity. Exposure of cells to this oligonucleotide led to a specific, dose-dependent decrease in RIα protein and to a significant inhibition in the rate of uPA internalization. We further demonstrate that treatment of melanoma cells with either H-89 or PKA RIα antisense oligonucleotides also resulted in a decreased internalization of two other ligands of LRP, activated α2M and lactoferin, indicating that PKA activity is associated with LRP. Finally, we demonstrate that PKA activity is also required for the internalization of transferrin, but not for the internalization of the epidermal growth factor or adenovirus 2, suggesting that in melanoma cells, PKA activity is not generally required for clathrin-mediated endocytosis, but is rather associated with specific internalization receptors.

Key words: Endocytosis, Protease receptor, uPAR, LRP/α2M-receptor, Protein kinase, PKA

INTRODUCTION

Urokinase-type plasminogen activator (uPA) is a serine protease that participates in proteolytic tissue remodeling by catalyzing the conversion of plasminogen into plasmin which, in turn, can degrade fibrin and glycoproteins of the extracellular matrix (for review see Vassalli et al., 1991; Danø et al., 1985). uPA is secreted as a single chain enzyme (sc-uPA) with low proteolytic activity that is cleaved into active two-chain uPA. Both forms of uPA bind with high affinity to the uPA receptor (uPAR), a 50-55 kDa glycoprotein that is anchored in the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety (Behrendt et al., 1995). uPA binding to uPAR is important in the regulation of cell surface plasminogen activation. It enhances uPA activity (Ellis et al., 1992) and removes uPA from the cell surface by receptor-mediated endocytosis. Many cell types internalize and degrade receptor-bound uPA slowly, but uPA complexed to the type 1 plasminogen activator inhibitor (PAI-1) with an accelerated rate (Cubellis et al., 1990; Jensen et al., 1990).

Internalization of uPA/PAI-1 complexes involves the cooperation of two receptors, the GPI-linked uPAR (Olson et al., 1992; Conese et al., 1995) and a transmembrane receptor of the low density lipoprotein (LDL) receptor family, namely the LDL receptor-related protein (LRP)/α2-macroglobulin (α2M) receptor (Nykjær et al., 1992; Herz et al., 1992). LRP is a multi-ligand receptor that also mediates the internalization of sc-uPA in the absence of PAI-1 (Kounnas et al., 1993), as well as the internalization of numerous other ligands (for review see Strickland et al., 1995), including proteases, protease/inhibitor complexes and lipoproteins. The receptor consists of an extracellular 515 kDa heavy chain containing the ligand-binding domains and a membrane spanning 85 kDa light chain (Herz et al., 1990). The cytoplasmic portion of the LRP light chain contains two repeats of the NPXY motif that targets the receptor to clathrin-coated pits for internalization (Chen et al., 1990).

The mechanism by which both receptors, uPAR and LRP, contribute to the internalization of uPA is not entirely understood. Kounnas et al. (1993) studied the internalization of sc-uPA by HepG2 cells and proposed that sc-uPA binds initially to uPAR followed by a ligand transfer to LRP and internalization. In a different experimental system, however, sc-uPA bound to soluble uPA was protected from binding to LRP and only after sc-uPA was converted to two-chain uPA and then complexed with PAI-1 did such uPA-PAI-1 complexes bind to uPAR and LRP and were efficiently internalized (Nykjær et al., 1994). In this regard, it has been shown that the LRP-mediated internalization of uPA-PAI-1 involves the internalization of uPAR itself (Conese et al., 1995).
Receptor-mediated endocytosis involves the sorting of membrane proteins into specialized regions of the plasma membrane and a sequence of regulated membrane fusion events (Lamaze et al., 1995; Robinson et al., 1996). Among the different pathways for endocytosis, clathrin-coated pit mediated endocytosis is a common, well-characterized mechanism that is energy dependent and regulated by small GTPases (Zerial and Stenmark, 1993; Schmid and Danke, 1995). Endocytosis depends also on the contribution of other signal transduction pathways. For example, the internalization of receptor tyrosine kinases, such as the epidermal growth factor (EGF) receptor and the insulin receptor, requires receptor phosphorylation and downstream signal transduction (Seaman et al., 1996). Further, apical endocytosis in polarized epithelial cells has been shown to be modulated by two pathways involving the serine/threonine protein kinases cAMP-dependent kinase (PKA) and protein kinase C (PKC) (Mostov and Cardone, 1995).

The process of uPA internalization has been extensively studied with regard to receptor ligand interactions; however, little is known about the mechanisms that regulate ligand-induced sorting of uPAR in the cell membrane and the process of uPA internalization, per se. Studies on the subcellular distribution of uPAR have demonstrated the receptor in different regions of the plasma membrane, including focal contacts, membrane ruffles and caveolae (Pöllänen et al., 1988; Myöhänen et al., 1993; Stahl and Mueller, 1995) and ligand binding is thought to recruit uPAR into an endocytic compartment. In contrast, LRP, like the LDL receptor (Goldstein et al., 1985), most likely moves spontaneously into coated pits and enters cells continuously, even in the absence of ligand.

The present studies were conducted to determine the intracellular signal transduction pathways involved in the endocytosis of uPA. We demonstrate here a role for PKA in the internalization of uPA and suggest that PKA activity is generally associated with LRP-mediated internalization, since it is also required for uPAR-independent LRP ligands.

### MATERIALS AND METHODS

#### Proteins, oligonucleotides and chemicals

Recombinant sc-uPA was provided by Dr Jack Henkin (Abbott Laboratories, Abbott Park, IL), amino-terminal fragment of uPA (ATF) was donated by Dr John Bogacki (American Diagnostica, Inc., Greenwich, CT) and recombinant soluble uPAR was a gift from Dr Steven Rosenberg (Chiron Corp., Emeryville, CA). Polyclonal rabbit anti-human uPAR antibody #399R was purchased from American Diagnostica Inc. (Greenwich, CT). The antibody fragment FabA8 against LRP was a gift from Drs Ivo Hor and Anton-Jan van Zonneveld (University of Amsterdam, The Netherlands). 125I-adenosine 2 virus was provided by Drs Erguang Li and Glen Nemerow (The Scripps Research Institute, La Jolla, CA). Human plasma 125I and human apotransferrin was obtained from Calbiochem (San Diego, CA) human lactoferrin from Sigma (St Louis, MO) and human 125I-EGF (>750 Ci/mmol) from Amersham (Arlington Heights, IL). The plasmid pGEX-KG-RAP coding for the human receptor associated protein (RAP) as a glutathione S-transferase (GST)-fusion protein was made available by Dr Joachim Herz (University of Texas, Southwestern Medical Center, Dallas). GST-RAP was expressed in E. coli and purified by the GST purification module from Pharmacia (Piscataway, NJ). A phosphorothioate modified antisense oligodeoxynucleotide for the RIIα subunit of human PKA with the sequence 5’-GGC-GGT-AGT-GCC-AGA-CTA-CTCAT-3’ (Yokozaki et al., 1993) was synthesized and HPLC-purified by Genset Corp. (La Jolla, CA). A 21mer random-sequence control oligonucleotide was made from a random mixture of all four nucleotides at every position. The protein kinase inhibitors bisindolylmaleimide I, calphostin C, genistein, H-89, K-252a, lavendustin A and staurosporine were obtained from Calbiochem (San Diego, CA). Myristoylated PKA specific inhibitor peptide (PKI) (14-22) amide and myristoylated protein kinase C (PKC) inhibitor peptide (19-27) were from Biomol (Plymouth Meeting, PA).

#### Cells and cell culture

The human melanoma cell line M21 was described previously (Mueller et al., 1990). M21 cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were harvested by brief exposure to versene.

#### Indirect immunofluorescence

M21 cells were harvested, washed and incubated for 1 hour on ice with polyclonal anti-uPAR IgG #399R (10 ng/ml) in 0.01 M phosphate, 0.145 M NaCl, pH 7.4, with 0.2% bovine serum albumin and 0.02% sodium azide. After extensive washing, cells were incubated with FITC-labeled goat anti-rabbit IgG antibody (Southern Biotechnology, Birmingham, AL) (15 µg/ml) for 45 minutes on ice. Alternatively, cells were incubated first with GST-RAP (10 µg/ml) followed by a polyclonal rabbit anti-GST antibody (15 µg/ml) for 1 hour on ice and finally with FITC-conjugated goat anti-rabbit IgG antibody. Labeled cells were analyzed by flow cytometry (FACSort, Becton Dickinson).

#### Preparation of activated α2M

Activated, receptor-binding competent α2M was generated by treatment with methylamine (Imber and Pizzio, 1981). Briefly, 1 mg α2M was dissolved in 0.5 ml 0.05 mM Tris-HCl, 0.15 M NaCl, pH 8.2. An equal volume of the same buffer containing 1.6 M MeNH2-HCl was added and allowed to react with α2M for 6 hours at 20°C. The solution was dialyzed against 0.01 M phosphate, 0.145 M NaCl, pH 7.4 and activated α2M was stored frozen.

#### Iodination of ligands

sc-uPA, lactoferrin or transferrin (100 µg) was iodinated with 1 mCi of Na125I (Amersham, Arlington Heights, IL) in the presence of 100 µg IODO-GEN reagent (Pierce, Rockford, IL) for 25 minutes on ice and then passed through a Sephadex G-25 Medium PD-10 column (Pharmacia, Piscataway, NJ) to remove free iodine. The specific activity was about 5,000 cpm/ng sc-uPA unless indicated otherwise, 8,500 cpm/ng lactoferrin and 9,500 cpm/ng transferrin. Labeling of 130 µg activated α2M with 2 mCi Na125I resulted in a specific activity of approximately 20,000 cpm/ng protein. The specific activity of 125I-adenosine 2 was 3×10⁵ cpm/virus particle.

#### Internalization assay

To determine the internalization rate of uPA, we used the inside/surface (Ins/Sur) plot technique, as described (Wiley and Cunnigham, 1982). Cells were transferred to 12-well Falcon plates (Becton Dickinson, Lincoln Park, NJ) at 3×10⁵ cells/well and allowed to grow for 24 hours. Medium was removed and replaced with RPMI 1640 containing 0.1% bovine serum albumin. 125I-sc-uPA (10 ng/well) was added to cell monolayers for various times at 37°C. After this incubation, plates were placed on ice and washed with ice-cold RPMI 1640, 0.1% bovine serum albumin. Surface-bound uPA was removed by acid-stripping with ice-cold 0.2 M acetic acid, pH 2.6, containing 0.1 M NaCl, for 10 minutes at 4°C. After further washing, cell monolayers were solubilized with 1 M NaOH for 1 hour. The radioactivity in the stripping solutions (surface) and the extracts (inside) was determined with a γ-counter. Nonspecific binding or uptake was assayed in the presence of a 200-fold excess of unlabeled uPA.
sc-uPA and these values were subtracted before the Ins/Sur ratios were calculated. When protein kinase inhibitors were used from stock solutions prepared in organic solvents (dimethylsulphoxide or ethanol), identical volumes of the organic solvent were added to control wells in the internalization assay. The inhibitors were added to the cells 20-30 minutes prior to addition of the radiolabeled ligands and kept at 37°C.

Antisense oligonucleotide treatment
Cells were grown to 40-50% confluency in 75 cm² tissue culture flasks. Oligonucleotides were added onto the washed cells at various concentrations in serum-free medium (Opti-MEM I, Gibco/BRL, Grand Island, NY) containing lipofectin reagent (Gibco/BRL) at 70 μg/ml. After 4 hours incubation at 37°C, 10 ml of RPMI 1640 medium with 20% fetal bovine serum was added to each flask. After incubation for three days at 37°C, cells were harvested, assayed for PKA protein by immunoblotting and used in internalization assays.

Cell lysates and immunoblot analysis
Cell pellets were washed two times with 0.01 M phosphate, 0.145 M NaCl, pH 7.4, and lysed for 30 minutes in 0.3 ml of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, and 0.2 mM PMSF. Lysates were separated by SDS-PAGE on a 14% polyacrylamide gel (Novex, San Diego, CA) and transferred to a nitrocellulose membrane. Membranes were blocked with Tris-buffered saline-Tween (TBS-T; 10 mM Tris-HCl, 140 mM NaCl, 2% Tween-20), containing 5% milk powder overnight at 4°C and incubated for 1 hour at room temperature with anti-PKA RIα primary antibody (Transduction Laboratories, Lexington, KY) at 1 μg/ml in TBS-T, 1% milk powder. After extensive washing, the membrane was incubated for 1 hour at room temperature with anti-mouse IgG antibodies coupled to horseradish peroxidase (Bio-Rad, Richmond, CA) at a dilution of 1:3,000 in TBS-T, 1% milk powder. After four washes, antigens were visualized using the enhanced chemiluminescent method (ECL kit from Amersham Life Science, Arlington Heights, IL).

RESULTS

Internalization of uPA by human melanoma cells is dependent on uPAR and LRP
Two receptors, the GPI-linked uPAR and the transmembrane LRP have been implicated in the internalization of uPA. M21 melanoma cells express both receptors as demonstrated by cell surface staining with anti-uPAR antibodies (Fig. 1a) and with the receptor-associated protein (RAP) (Fig. 1b), a high affinity ligand for LRP (Williams et al., 1992). M21 cells efficiently internalized 125I-labeled sc-uPA (Table 1) and we tested whether uPA binding to uPAR and/or LRP was required in this process. To implicate uPAR, we tested the effect of an excess of either the amino-terminal fragment of uPA (ATF) or soluble uPAR on the internalization of uPA. ATF blocks binding of uPA to uPAR, but not to LRP (Nykjær et al., 1992; Kounnas et al., 1993) and soluble uPAR competes with uPA binding to cell surface uPAR and also prevents sc-uPA from binding to LRP (Nykjær et al., 1994). A 500-fold excess of ATF or a 125-fold excess of soluble uPAR significantly reduced sc-uPA on the surface and on the inside of M21 cells (Table 1), indicating that binding to uPAR is a requirement for the efficient internalization of sc-uPA by M21 cells. Surface and inside ligand in the presence of ATF is only a small percentage of ligand bound in the absence of ATF, but may represent sc-uPA binding and internalizing independent of uPAR, i.e. solely mediated by LRP.

To demonstrate the involvement of LRP in the internalization of sc-uPA by M21 cells, we performed internalization experiments in the presence of excess RAP, which prevents binding and internalization of all known LRP ligands (Williams et al., 1992) or FabA8, an antibody fragment that specifically blocks the uPA binding site of LRP (Horn et al., 1995). Both RAP and FabA8 did not interfere with surface binding of sc-uPA, but inhibited the uptake into the cells (Table 1). In this regard, RAP was somewhat more efficient than FabA8, probably since it binds with higher affinity to LRP than FabA8. RAP can also inhibit ligand binding to other internalization receptors of the LDL receptor family like the glycoprotein 330 or the very low density lipoprotein receptor (Kounnas et al., 1992; Battey et al., 1994). Therefore, it is possible that in addition to LRP other receptors from the LDL receptor family can function as the transmembrane receptor in the internalization of uPA by M21 cells.

Internalization of uPA requires PKA activity
To study the mechanisms underlying the receptor-mediated internalization of uPA, we tested whether protein kinase activity was involved. It has been reported that uPAR is tightly associated with nonreceptor tyrosine kinase activity of the src-family and that ligation of uPAR leads to the activation of tyrosine kinases (Bohuslav et al., 1995; Resnati et al., 1996). Therefore, we considered an involvement of tyrosine kinases in the internalization of uPA. However, two potent inhibitors of tyrosine kinases, genistein and lavendustin A, had no effect on the internalization of uPA by M21 cells (Fig. 2), making it unlikely that tyrosine kinases are involved in the internalization of uPA. In contrast, the broad spectrum inhibitors of serine/threonine specific kinases, staurosporine (Meyer et al., 1989) and K-252a reduced the internalization of uPA significantly, with staurosporine demonstrating maximal inhibition at 3 μM and K-252a at 15 μM (Fig. 2). The internalization of uPA was also inhibited in the presence of 25 μM H-89 (Fig. 2) that

Table 1. Internalization of uPA by M21 cells requires binding to uPAR and LRP

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<thead>
<tr>
<th>Addition</th>
<th>15 minutes</th>
<th>30 minutes</th>
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<tr>
<td></td>
<td>Surface cpm</td>
<td>Inside cpm</td>
<td>Surface cpm</td>
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<tr>
<td>Control</td>
<td>978±21</td>
<td>129±11</td>
<td>2153±145</td>
</tr>
<tr>
<td>ATF (20 nM)</td>
<td>157±13</td>
<td>81±10</td>
<td>174±15</td>
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<tr>
<td>Soluble uPAR (5 nM)</td>
<td>86±8</td>
<td>89±10</td>
<td>139±13</td>
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<tr>
<td>GST-RAP (150 nM)</td>
<td>1054±43</td>
<td>95±10</td>
<td>2785±208</td>
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<tr>
<td>FabA8 (100 nM)</td>
<td>1009±85</td>
<td>96±9</td>
<td>2630±201</td>
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M21 cells were incubated with 40 pM 125I-sc-uPA in the absence (control) or presence of the indicated additions. ATF was added 30 minutes prior to incubation with 125I-sc-uPA. Soluble uPAR, GST-RAP or FabA8 were added immediately prior to incubation with 125I-sc-uPA.
inhibits PKA approximately 1,000-fold more efficiently than PKC or other serine kinases (Chijiwa et al., 1990). Thus, inhibition by H-89 suggests a role for PKA in the internalization of uPA by M21 cells. However, since the Ki of H-89 for purified PKA has been reported to be 48 nM (Chijiwa et al., 1990) at the concentrations effective in M21 cells, H-89 may also be affecting other kinases. No effect on the internalization of uPA by M21 cells was achieved at a 10 μM concentration of bisindolylmaleimide I or calphostin C (Fig. 2), both specific inhibitors of PKC, which may inhibit PKA at much higher concentrations (Toullec et al., 1991; Jarvis et al., 1994). At the concentrations used, neither protein kinase inhibitor affected uPA binding to the cell surface or cell viability.

The effect of kinase inhibitors on the internalization of uPA suggested a role for PKA, but not PKC, activity. To confirm this contention, we used specific autoinhibitory pseudosubstrate peptides against PKA and PKC, respectively, which hold the kinases in the inactive state by occupying their substrate binding cavity (Glass et al., 1989). Incubation of M21 cells with the pseudosubstrate peptide PKI (14-22), directed against the catalytic domain of PKA, which may inhibit PKA at much higher concentrations (Toullec et al., 1991; Jarvis et al., 1994). At the concentrations used, neither protein kinase inhibitor affected uPA binding to the cell surface or cell viability.

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PKA is a tetrameric protein that consists of two regulatory and two catalytic subunits. Different isoforms of the regulatory subunit, termed RIα RIβ RIIα and RIIβ have been identified (Francis and Corbin, 1994) that differ in subcellular and tissue distribution. The RI isoform of PKA is found throughout the cytoplasm and we therefore tested whether the activity of this isoform might be involved in internalization. A phosphoro-thioate modified antisense oligonucleotide against the RIα subunit was shown to abolish PKA-I activity in several tumor cell lines (Yokozaki et al., 1993; Nesterova and Cho-Chung, 1995). Consequently, we used this antisense oligonucleotide to block RIα expression in M21 cells. Cells were exposed for three days to the oligonucleotide at different micromolar concentrations and the effect on RIα expression was monitored by western blot analysis. The antisense treatment resulted in a marked, dose-dependent reduction of RIα protein, as compared to cells treated with a random sequence oligonucleotide (Fig. 4). Because long term exposure to oligonucleotides at 20 μM
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Concentration resulted in some cell death, the internalization rate for uPA was determined with M21 cells treated for three days with either 5 or 10 μM oligonucleotides. Under these conditions, treatment with the RIα antisense oligonucleotide significantly reduced the internalization of uPA, whereas the random sequence control oligonucleotide had no effect (Fig. 5). Together, these data demonstrate that PKA activity and specifically the RIα subunit of PKA, is required for the receptor-mediated internalization of uPA.

**PKA activity is required for the internalization of different LRP ligands**

In order to determine whether PKA activity was specifically involved in the internalization of uPA, we determined the effect of PKA inhibition on the cellular uptake of proteins that are thought to require only binding to LRP for internalization. To this end, we studied two LRP ligands, methyamine activated α2M (Ashcom et al., 1990) and lactoferrin (Willnow et al., 1992). Both proteins were rapidly internalized by M21 cells and their internalization was inhibited in the presence of RAP (data not shown). In the presence of the PKA inhibitor H-89 or after pretreatment of the cells with the antisense oligonucleotide directed against the RIα subunit of PKA, internalization of both α2M and lactoferrin was markedly reduced (Fig. 6). Thus, PKA activity is involved not only in the internalization of uPA, but also in the internalization of other LRP ligands.

**Fig. 3.** Effect of a pseudosubstrate peptide for PKA on the internalization of uPA: M21 cells were incubated with 40 pM 125I-sc-uPA for 70 minutes at 37°C in the presence of various concentrations of either the myristoylated pseudosubstrate PK1 (14-22) or PKA (■) or the PKC-specific pseudosubstrate peptide PKC (19-27). The specific activity was 9,000 cpm/ng sc-uPA. Surface-bound and internalized radiolabel was determined, as described in Materials and Methods. Values represent the mean of two experiments each performed in duplicate; the standard error of the mean values were less than 10%.

**Fig. 4.** Western blot analysis of PKA RIα expression: M21 cells were treated with oligonucleotides, as described in Materials and Methods. Total cell lysates (15 μg protein per lane) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the anti-PKA RIα antibody, followed by the secondary antibody, as described. Shown are human fibroblasts as positive control (A), M21 cells treated with 5 μM random oligonucleotide (B), or with 20 μM (C), 10 μM (D), 5 μM (E), or 1 μM (F) of RIα antisense oligonucleotide.

**Fig. 5.** Effect of RIα antisense oligonucleotide on uPA internalization: M21 cells were treated with 5 or 10 μM RIα antisense oligonucleotide or with 10 μM random sequence control oligonucleotide, as described in Materials and Methods. Internalization of 40 pM 125I-sc-uPA was determined for 60 minutes at 37°C on oligonucleotide treated or untreated control cells. Each bar represents mean and s.d. of two independent experiments performed in quadruplicate.

**Fig. 6.** Role of PKA in the internalization of different LRP ligands: internalization of 50 nM methyamine activated 125I-α2M (black columns) or 2 nM 125I-lactoferrin (white columns) by M21 cells was determined for 60 minutes at 37°C. (A) Internalization in the presence of 40 μM H-89; and (B) internalization by cells treated with either 5 μM RIα antisense oligonucleotide or 5 μM random sequence control oligonucleotide. Each bar represents mean and s.d. of two independent experiments performed in duplicate.
We also addressed whether in M21 cells PKA activity is involved in processes of receptor-mediated internalization that are independent of LRP. We tested the effect of the PKA inhibitor PKI (14-22) on the uptake of transferrin and EGF, two typical ligands for clathrin-coated pit-mediated internalization (Damke et al., 1994), as well as adenovirus 2, which upon binding to an integrin (Wickham et al., 1993), is internalized by a clathrin-dependent mechanism (Drs E. Li and G. Nemerow, personal communication). The internalization of transferrin into M21 cells decreased in the presence of PKI (14-22), whereas the internalization of EGF and adenovirus 2 was not affected (Fig. 7). In contrast to the uptake of uPA by M21 cells, the internalization of transferrin was also inhibited by the pseudosubstrate for PKC (data not shown). These data indicate that clathrin-dependent internalization encompasses multiple signaling pathways and that internalization by some internalization receptors, such as LRP and the transferrin receptor, but not by others, is associated with PKA activity.

**DISCUSSION**

Evidence is provided here for a role of PKA activity in the receptor-mediated internalization of uPA by human melanoma cells. In this regard, we demonstrate that the internalization of uPA is inhibited in the presence of either the protein kinase inhibitor H-89 that preferentially inhibits PKA or the inhibitory pseudosubstrate peptide for PKA, PKI (14-22). We further demonstrate that treatment with a specific antisense oligonucleotide blocked the expression of the RIα subunit of PKA and also inhibited internalization of uPA. This implies a role for the RI isoform of PKA in internalization of uPA because it has been shown for several human cancer cell lines that antisense-inhibition of RΙα resulted in the elimination of the RIα subunit and the RIα-containing holoenzyme PKA-I with a concomitant increase in PKA-II (Yokozaki et al., 1993; Nesterova and Cho-Chung, 1995).

PKA activity has been implicated previously in other cellular processes involving controlled vesicular transport. For example, apical endocytosis of cell-surface bound ricin in polarized MDCK cells was stimulated upon activation of adenyl cyclase and an increase in cAMP (Eker et al., 1994). Similarly, activation of PKA by increasing intracellular cAMP levels resulted in increased transport of influenza hemagglutinin from the trans-Golgi network to the apical surface of epithelial cells (Pimplikar and Simons, 1994). The apically directed transcytosis in MDCK cells was reported to involve a signaling pathway in which a heterotrimeric G protein containing the Gsα subunit activates adenyl cyclase which, in turn, activates PKA (Hansen and Casanova, 1994). In the internalization of uPA, PKA activity may also be modulated by heterotrimeric G-proteins. In this regard, we found that mastoparan, an activator of heterotrimeric G-proteins, which preferentially acts on the Gsα subunit that inhibits adenyl cyclase rather than on the stimulatory Gi (Higashijima et al., 1990), also inhibited the internalization of uPA (L. Goretzki and B. M. Mueller, unpublished observation) presumably by decreasing the level of intracellular cAMP.

The internalization of uPA was not inhibited by the specific PKC inhibitors bisindolylmaleimide I and calphostin C or by a specific pseudosubstrate peptide for PKC. This finding was unexpected, because PKC has been implicated in several other internalization models. Thus, PKC activation has been shown to increase the rate of internalization of the transferrin receptor (Eichholtz et al., 1992). Integrin dependent endocytosis of vitronectin is stimulated upon PKC activation and inhibited by calphostin C (Panetti et al., 1995). The internalization of GPI-linked proteins, such as the folate receptor (Smart et al., 1994) and CD59 on lymphocytes (Deckert et al., 1996) was also shown to be regulated by PKC. PKC inhibitors vary in their specificity for different PKC isoforms. For example, the pseudosubstrate peptide PKC (19-27) inhibits specifically PKCa and PKCb Therefore, our data do not rule out the possibility that any isoform of PKC is involved in the internalization of uPA and that a PKC-dependent signaling pathway may function in either a parallel or a cooperative manner in addition to a PKA-dependent pathway. In this regard, it has been shown that PKA can modulate the activity of conventional and atypical PKC isoforms (Wooten et al., 1996; Anderson and Breckon, 1991) and that a cross-talk between two G protein-coupled signal transduction pathways, involving PKA and PKC, exists in COS-7 cells (Liu and Simon, 1996).

Internalization of GPI-linked proteins has been described to occur in caveolae (Anderson, 1993; Parton et al., 1994) or, in cells that lack caveolae, in glyco-lipid rich domains of the cell membrane (Deckert et al., 1996). We have previously shown that the GPI-linked uPAR on melanoma cells is localized in caveolae (Stahl and Mueller, 1995) and considered that the

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**Fig. 7.** Effect of PKI (14-22) on the internalization of transferrin, EGF and adenovirus 2: M21 cells were incubated at 37°C in the absence or presence of 40 μM myristoylated pseudosubstrate PKI (14-22) for PKA with 100 pM 125I-transferrin (Tf), 2 nM 125I-EGF or 7×10⁷ viral particles of 125I-adenovirus 2 (Ad2). Transferrin and EGF were incubated with M21 cells for 60 minutes and Ad2 with M21 cells for 20 minutes. Values represent the mean ± s.d. of two experiments performed in duplicate.
cellular uptake of uPA/uPAR may involve caveolae-mediated endocytosis. Here, we demonstrate that melanoma cells, like other cell types (Nykjær et al., 1992; Herz et al., 1992; Kounnas et al., 1993), require two receptors, uPAR and LRP, for the efficient internalization of sc-uPA and uPA-PAI-1 complexes. This suggests a clathrin coated pit-mediated pathway for the internalization of uPA because LRP, like other LDL-receptor family members, is targeted to clathrin coated pits (Chen et al., 1990). Furthermore, caveolae-mediated endocytosis has been shown to be regulated by PKC. Activators of PKC, such as phorbol-12-myristate-13-acetate, inhibit the invagination of caveolae and the cellular uptake of folate bound to the GPI-linked folate receptor (Smart et al., 1994). Specifically, the receptor-mediated uptake of folate has been shown to be regulated by a population of PKCα molecules that resides in the membrane of caveolae (Smart et al., 1995). Taken together, these reports and our current data on the requirement for LRP and the lack of an effect of PKC inhibitors, make it seem unlikely that uPA/uPAR is internalized by caveolae.

We demonstrate here that the internalization of uPA by human melanoma cells requires binding of uPA to two receptors, the GPI-linked uPAR and the transmembrane LRP. When we extended our studies to the internalization of additional LRP ligands, we found that PKA was also involved in the internalization of two proteins, α2M and lactotransferrin, that are internalized by LRP independent of uPAR, indicating that PKA activity is not specifically associated with uPAR, but rather appears to be required for the internalization of all LRP ligands. This suggests the possibility that LRP itself is a target of PKA. We have, however, no evidence for LRP being phosphorylated by PKA and while there are several serine residues in the cytoplasmic domain of LRP, the PKA consensus sequence LRRASGL is not realized. Another possibility is that LRP is involved in the modulation of PKA activity. Although LRP has not been directly demonstrated to be associated with G-proteins or protein kinases, it has been reported that lactotransferrin binding to LRP results in an increase of intracellular Ca2+ and inositol phosphates (Misra et al., 1994). This response can be inhibited by pertussis toxin (Misra et al., 1994), indicating that LRP is associated with a pertussis toxin-sensitive heterotrimeric G-protein. If LRP is in fact directly associated with a G-protein, ligation of LRP may modulate PKA activity and thereby regulate LRP-mediated internalization. The idea that PKA activity is associated with specific internalization receptors is supported by our demonstration that in M21 cells, the internalization of LRP ligands and transferrin, but not the internalization of EGF and adenovirus 2, requires PKA activity. Whether ligand binding to specific internalization receptors modulates PKA activity directly or whether regulatory factors along the endocytic pathway require PKA activity remains the subject of further studies.

In summary, we demonstrate here that the inhibition of PKA activity and blocking of RIIα expression results in a decreased internalization of uPA. Since PKA activity is also required for the internalization α2M and lactotransferrin, PKA may be part of a signaling pathway downstream of LRP.

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