# Interaction of Urokinase-Type Plasminogen Activator with Its Receptor Rapidly Induces Activation of Glucose Transporters†

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ABSTRACT: The interaction of urokinase-type plasminogen activator (u-PA) or of u-PA amino-terminal fragment (u-PA-ATF) with the cell surface receptor (u-PAR) was found to stimulate an increase of glucose uptake in many cell lines, ranging from normal and transformed human fibroblasts, mouse fibroblasts transfected with human u-PAR, and cells of epidermal origin. Such increase of glucose uptake reached a peak within 5−10 min, depending on the cell line, and occurred through the facilitative glucose transporters (GLUTs), since it was inhibited by cytochalasin B. Each cell line showed a specific mosaic of glucose transporter isoforms, GLUT2 being the most widespread and GLUT1 the most abundant, when present. u-PAR stimulation was followed by translocation of GLUT1 from the microsomal to the membrane compartment, as shown by both immunoblotting and immunofluorescence of sonicated plasma membrane sheets and by activation of GLUT2 on the cell surface. Both translocation and activation resulted inhibitable by protein−tyrosine kinase inhibitors and independent of downregulation of protein kinase C (PKC). The increase of intracellular glucose was followed by neosynthesis of diacylglycerol (DAG) from glucose, as previously shown. Such neosynthesis was completely inhibited by impairment of facilitative GLUT transport by cytochalasin B. DAG neosynthesis was followed by activation of PKC, whose activity translocated into the intracellular compartment (PKM), where it probably phosphorylates substrates required for u-PAR-dependent chemotaxis. Our data show that u-PAR-mediated signal transduction, related with u-PA-induced chemotaxis, involves activation of tyrosine kinase-dependent glucose transporters, leading to increased *de novo* DAG synthesis from glucose, eventually resulting in activation of PKC.

In addition to its function in the fibrinolytic system, several lines of evidence indicate that interaction of the urokinase-type plasminogen activator (u-PA)[1] with its cellular receptor (u-PAR) elicits a complex series of events that range from chemotaxis and chemokinesis (Fibbi et al., 1988; Del Rosso et al., 1992, 1993; Odekon et al., 1992; Anichini et al., 1994; Busso et al., 1994) to cell multiplication (Kirchheimer et al., 1987, 1989; Rabbani et al., 1990, 1992; He et al., 1991; Anichini et al., 1994), differentiation (Nusrat & Chapman, 1991), and autocrine secretion of u-PA (Fibbi et al., 1990). In most cases, the effects following u-PA/u-PAR interaction do not require the presence of the catalytic site of the enzyme, DNA duplication being the only exception (though there is evidence to the contrary, at least in one cell line) (Rabbani et al., 1990, 1992). Thereby, the interaction can be considered similar to that occurring between polypeptide chemotactic/growth factors and their membrane receptors. By its very nature, such interaction requires transduction at the level of the cell membrane. Some papers have recently addressed this topic: an association of u-PAR with a not yet identified 38 kDa protein that undergoes u-PA-dependent tyrosine phosphorylation has been described (Dumler et al., 1994); recent data from Resnati et al. (1996) indicate u-PA-dependent activation of p56/p59$^{hck}$, a u-PAR-associated member of Src family of tyrosine kinases, in THP-1 cells; urokinase plasminogen activator receptor, $\beta$2-integrins, and Src kinases have been shown to form a single receptor complex in human monocytes (Bohuslav et al., 1995). These data support the hypothesis of an early activation of a tyrosine kinase following u-PA/u-PAR interaction. Cytokeratins 8 and 18 have been shown to be specifically phosphorylated on serine residues by u-PAR-delivered signals (Busso et al., 1994), indicating the activation of a protein kinase C isoform. Consistently, in cell lines of epidermal and fibroblast origin challenged with either u-PA or its amino-terminal fragment (ATF), we have shown an increase of diacyglycerol (DAG) formation resulting from *de novo* synthesis from glucose, indpendent of inositol lipid metabolism and intracellular calcium release (Del Rosso et al., 1993; Anichini et al., 1994). DAG can be synthesized *de novo* from the glycolytic intermediate dihydroxyacetone phosphate and from glycerol 3-phosphate by pancreatic islets in response to insulin and human neutrophils during phagocytosis (Farese et al., 1986; Peter-Riesch et al., 1988; Rossi et al., 1991). Such elevated DAG *de novo* synthesis is secondarily due to increased formation of precursors derived from glucose metabolism, that is, to the "mass effect" of insulin-stimulated intracellular glucose increase (Lee et al., 1989a,b). The glucose uptake across the membrane of mammalian cells is facilitated by a family of glucose transport proteins (GLUT), which are the

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[1] Abbreviations: ATF, amino-terminal fragment of u-PA; DAG, diacylglycerol; ECL, enhanced chemiluminescence; GLUT, glucose transporter; InsPs, inositol phosphates; PKC, protein kinase C; PKM, protein kinase M; PTK, protein tyrosine kinase; u-PA, urokinase-type plasminogen activator; u-PAR, urokinase-type plasminogen activator receptor.

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products of distinct genes and are expressed in a highly controlled tissue-specific fashion [for a review, see Gould and Holman (1993)]: these transporters allow the movement of glucose across the plasma membrane down its chemical gradient either into or out of the cells. While some tissues express a single GLUT, others show a mosaic of transporter proteins: adipocytes express two transporter isoforms, the ubiquitous glucose transporter GLUT1 and the fat/muscle-specific, insulin-responsive glucose transporter GLUT4. Abundant evidence indicates that the predominant mechanism by which an acute insulin treatment regulates hexose flux involves a redistribution of glucose transport proteins from an intracellular (mainly microsomal) pool to the plasma membrane (James et al., 1989), while chronic insulin treatment increases hexose flux by a mechanism involving both increased expression and translocation of GLUT1 (Tordjman et al., 1990). In this paper we investigated whether the rapid activation of DAG synthesis from glucose occurring following u-PA or u-PA-ATF treatment of u-PAR-expressing cells could be related to activation or membrane translocation of glucose transporter proteins able to increase the intracellular glucose mass, within a time span compatible with transduction of the external signal into cellular functions. We show that addition of uPA or u-PA-ATF to many cell lines results in a noticeable increase of glucose uptake that, for some cell lines, is related to translocation of GLUT1 from an intracellular compartment to the membrane or to activation of GLUT2. Moreover, we provide evidence that DAG neosynthesis and the following activation of protein kinase C are strictly dependent on u-PAR-stimulated glucose uptake in all cell lines examined.

## MATERIALS AND METHODS

*Antibodies and Antisense Oligonucleotides.* Polyclonal rabbit anti-GLUT1, anti-GLUT2, anti-GLUT3, and anti-GLUT4 glucose transporter antibodies were purchased from East Acres (Southbridge, MA). All the antibodies were used for both immunoblotting and immunofluorescence. Peroxidase-conjugated anti-mouse and anti-rabbit IgGs, as well as fluorochrome-conjugated antibodies for immunofluorescence, were from Sigma Immuno Chemicals (St. Louis, MO). The mouse anti-u-PA amino-terminal fragment (ATF) mAb 5B4 was a kind gift of Dr. M. L. Nolli (Dow-Lepetit SpA, Milan). The mouse mAb R3 against the u-PA-binding domain of u-PAR was a kind gift of Dr. F. Blasi (Ronne et al., 1991). Both 5B4 and R3 mAbs were purified by G-protein affinity chromatography (Pharmacia, Sweden). To inhibit u-PAR gene expression, an 18-mer anti-messenger oligodeoxyribonucleotide (aODN) phosphodiester has been used, as previously described (Quattrone et al., 1995a,b). The anti-u-PAR aODN (5′-CGG CGG GTG ACC CAT GTC-3′) was designed to encompass the translation start site of the targeted mRNA, corresponding to residues 44−61 of the human u-PAR gene cDNA sequence (Roldan et al., 1990). Such aODN was protected against exonuclease cleavage by a double substitution at the 3′-end and 5′-end with phosphorothioate residues (Genosys). A scrambled ODN sequence (ssODN) with the same phosphorothioate substitutions was used as a control. Both ODN uptake and stability were enhanced by combining ODNs with a cationic liposome, namely, DOTAP (Boehringer Mannheim, Mannheim, Germany), as described (Quattrone et al., 1995a,b). DOTAP-combined ODNs were applied to the culture medium containing 10% heat-inactivated FCS (GIBCO, Gaithersburg, MD) at the final concentrations of 10 $\mu$M ODNs and 13 $\mu$M DOTAP. DOTAP-combined ODNs half-life and cellular toxicity were determined previously for each cell line.

*Ligands.* The following reagents were provided by Dow-Lepetit SpA (Milan), courtesy of Dr. M. L. Nolli: human two-chain u-PA and the catalitically active fragment of human u-PA, LMW u-PA. Two-chain u-PA was also purchased from Serono (Rome). All forms of u-PA were purified by affinity chromatography on Sepharose CH-4B (Pharmacia) substituted with *p*-aminobenzamidine (Holmberg et al., 1970), as previously described (Del Rosso et al., 1985). Both two-chain u-PAs and LMW u-PA resulted pure on polyacrilamide gel electrophoresis performed after the purification step (not shown). u-PA-ATF, a kind gift of Dr. J. Henkin (Abbott Laboratories, Chicago, IL), resulted electrophoretically pure without further purification (not shown).

*Cells.* The established cell line NCTC 2544 (epithelial cells from human epidermis), human adult fibroblasts from skin biopsies, the VA-13 cells (SV40-transformed human embryonic lung fibroblasts), and LB6 clone 19 mouse fibroblasts (transfected with the human u-PAR gene) were cultured in Dulbecco's-modified minimal essential medium (DMEM), supplemented with 10% fetal calf serum, in an atmosphere of 5% $CO_2$−95% air at 37 °C. All these cell lines were chosen on the basis of the existence of u-PARs on their surface and of biological effects elicited by u-PA/u-PAR interaction (Del Rosso et al., 1992; Anichini et al., 1994; Roldan et al., 1990; Quattrone et al., 1995a,b).

*Immunoblotting and Immunofluorescence.* SDS−polyacrylamide gel electrophoresis (PAGE) and Western blots were carried out essentially as described earlier (Fibbi et al., 1990). Antibodies were used at the following dilutions for immunoblotting: anti-GLUT1, 1/2000; anti-GLUT2, 1/1000; anti-GLUT3, 1/1000; anti-GLUT4, 1/500; anti-rabbit peroxidase-conjugated IgG, 1/2000. Specific bands were revealed by an ECL-based detection system (Amersham) using Hyperfilm-ECL (Amersham). Molecular weights were calculated from the position of markers electrophoresed in parallel lanes and stained with Coomassie blue. Immunofluorescence was performed according to a standard protocol. In experiments aiming to show the translocation of glucose transporter molecules from intracellular compartments to the surface membrane, immunofluorescence was performed on plasma membrane sheets as described by Fingar and Birnbaum (1994). Cells grown to confluence on coverslips (both unstimulated and stimulated for 10 min with u-PA or ATF) were washed twice in ice-cold buffer A (100 mM NaCl, 50 mM Hepes, pH 7.3) and once in ice-cold buffer B (100 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 $\mu$M leupeptin, 2 $\mu$g/mL trypsin inhibitor, 0.5 mM benzamidine, 20 mM Hepes, pH 7.2) and then sonified in buffer B for 3 s using a sonifier with a microtip placed 1 cm above the coverslip. Adherent plasma membrane sheets were washed twice in ice-cold buffer B, fixed in 4% paraformaldehyde for 10 min, and processed for immunofluorescence using anti-GLUT antisera and fluorochrome-conjugated secondary antibodies, according to a standard protocol. Briefly, cell plasma membranes were washed three times with PBS, incubated for 10 min in 0.1% bovine serum albumin (BSA) in PBS, and then incubated for 45 min with primary antibodies, diluted in BSA (0.1% in PBS) as reported above. After three washings of 5 min with BSA (0.1% in PBS), coverslips were incubated for 30 min with fluorochrome-conjugated secondary antibod-

ies, washed again, and mounted on light microscope slides for observation.

*Hexose Uptake.* Cell were grown on confluence in 3 cm diameter tissue culture dishes (NUNC-Intermed, Denmark). Twenty-four hours before the experiment cells were serum-starved and added with 5.5 mM 2-deoxy-D-glucose (2-DOG). Cell monolayers were then washed two times with Hepes buffer and incubated in the same buffer (1 mL/dish) at 37 °C with or without (control conditions) u-PA or u-PA-ATF for various times. At fixed time intervals 2-deoxy-D-[2,6-$^3$H]glucose (1 $\mu$Ci/mL) was added for 4 min, and cells were incubated at 37 °C. The incubation medium was poured off, dishes were placed on ice, and cells were then rapidly washed four times with ice-cold Hepes buffer containing 5.5 mM DOG, and the radioactivity was extracted with 1 mL of 0.2 M NaOH. Nonspecific association of $^3$H-DOG with cells was evaluated by measuring glucose uptake in the presence of 10 $\mu$M cytochalasin B, a potent inhibitor of transport (Bloch, 1973). Specific GLUT-dependent glucose uptake was obtained by subtracting nonspecific from specific uptake.

*Subcellular Fractionation.* Confluent cell monolayers were fractionated as described (Simpson et al., 1983). Briefly, the cells were washed twice in a 20 mM Tris-HCl−1 mM EDTA−225 mM sucrose buffer, pH 7.4, resuspended in 1 mL of the same buffer, and homogenized with 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 16000*g* for 15 min, and the supernatant was saved for preparation of microsomal membrane fractions. The initial pellet was resuspended and recentrifuged once before being resuspended in 5 mL of the original buffer, applied to a 1.12 M sucrose cushion containing 20 mM Tris-HCl−1 mM EDTA, and centrifuged at 101000*g* for 70 min. The plasma membrane, collected at the interface, was resuspended in 0.8 mL of buffer and centrifuged at 48000*g* for 45 min. The pellet was resuspended in 50 $\mu$L of buffer, repelleted, and stored frozen for further analysis. The initial supernatant was centrifuged at 212000*g* for 70 min, in order to collect total microsomal membranes; the pellet was resuspended in 50 $\mu$L of buffer and stored frozen. The presence and the specific isotype of the glucose transporter were evaluated on aliquots of each fraction, after determination of the protein content.

*Determination of Inositol Phosphates and 1,2-Diacylglycerol (1,2-DAG).* In experiments involving measurements of inositol phosphates (InsPs), the day after plating cell monolayers were prelabeled to equilibrium (48 h) with myo-[2-$^3$H]inositol, following the method described in Lacal et al. (1987). After radiolabeling, the cultures were washed and fresh serum-free medium was added for 6 h. After this incubation under serum-free conditions, the medium was replaced with a fresh one containing 10 mM LiCl. Cells were then stimulated for 20 min with u-PA or ATF, lipids were extracted by methanol−chloroform extraction, and InsPs were separated by ion-exchange chromatography as described (Del Rosso et al., 1993; Anichini et al., 1994). To study *de novo* synthesis of DAG from glucose, confluent cell monolayers were pulse-labeled for 4 h with [$^3$H]glucose or [$^3$H]glycerol and stimulated with u-PA or ATF in the presence of label; lipids were extracted by methanol−chloroform, and DAG was separated by thin-layer chromatography, as described in Lacal et al. (1987), using commercial lipids (Sigma) as reference standards. Results of second messenger measurements were expressed as percentage of variation in each analyzed metabolite over that without stimulation. To minimize error due to variability in cell number, labeling procedure, and extraction efficiency, the ratios DAG/phospholipids and inositol phosphates/phosphoinositides were considered in making calculations. This procedure of result normalization has proven reliable under a variety of experimental conditions involving second messenger measurement (Price et al., 1989; Del Rosso et al., 1993; Anichini et al., 1994).

Table 1

| cell line | u-PAR[a] | $K_d$[b] | [u-PA][c] | % increase[d] |
|---|---|---|---|---|
| human fibroblasts | 130[e] | 1.0[e] | 1.84 | 170 |
| LB6 clone 19 | 25[f] | 10[f] | 1.84 | 145 |
| NCTC | 11[g] | 1.3[g] | 0.92 | 250 |
| VA-13 | 175[h] | 4[h] | 3.68 | 230 |

[a] Number of receptors for urokinase-type plasminogen activator per cell × $10^{-3}$. [b] Value of the dissociation constant (nM). [c] uPA concentration (nM) able to induce the maximal increase of glucose uptake. [d] Maximal percent increase of glucose uptake after 5 min of stimulation. [e] Anichini et al., 1994. [f] Roldan et al., 1990. [g] Del Rosso et al., 1992. [h] Quattrone et al., 1995a,b.

*Determination and Cellular Localization of Protein Kinase C (PKC) Activity.* PKC activity was measured by a modification of the method described by Lee et al. (1989a,b). Both control and u-PA-challenged cells were washed twice with $Ca^{2+}$- and $Mg^{2+}$-free PBS and twice with buffer A [50 mM Tris-HCl, pH 7.5, containing 0.3% (w/v) $\beta$-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 50 $\mu$g/mL phenylmethanesulfonyl fluoride, 10% benzamidine, and 0.33 M sucrose], scraped from the dishes with 5 mL of the same buffer, and then homogenized with a glass−glass Dounce homogenizer for 30 strokes. The soluble fraction obtained after centrifugation at 100000*g* for 30 min was retained as cytosolic extract. The pellets were washed by resuspension in 5 mL of buffer B (buffer A without sucrose) and centrifuged again. The washed pellet was resuspended in 4 mL of buffer B with 1% Triton-X100 and homogenized again. After a 30 min incubation the resuspended solution was recentrifuged and the soluble fraction was collected as membrane extract. Aliquots of both cytosolic and membrane extracts were assayed for PKC activity by a PKC enzyme assay system (Amersham Life Science, England), according to the instructions of the manufacturer.

## RESULTS

*Effects of u-PA and u-PA-ATF on Glucose Transport.* The dose dependence of 2-DOG uptake under the stimulation of u-PA indicated that the most efficient concentration was different for each cell line and was similar to the $K_d$ of u-PA/u-PAR interaction reported in the literature (Table 1), LB6 clone 19 cells being the only exception. Following stimulation of cell monolayers with the most efficient concentrations of u-PA or u-PA-ATF, 2-DOG uptake was increased by 1.5−3.5-fold with a peak increase at 5−10 min, depending on the cell line under study (Figure 1). The increase of glucose uptake was of the facilitative type since it was inhibited by 10 $\mu$M cytocalasin B (Figure 2). Both preparations of two-chain u-PA described under Materials and Methods gave similar results on DOG uptake, while LMW-u-PA resulted completely ineffectual (not shown). Though the transduction mechanism leading to membrane recruitment and activation of glucose transporter in insulin-dependent and non-insulin-dependent cells is still a matter of investigation, it has been recently shown that interleukin-3 facilitates glucose transport

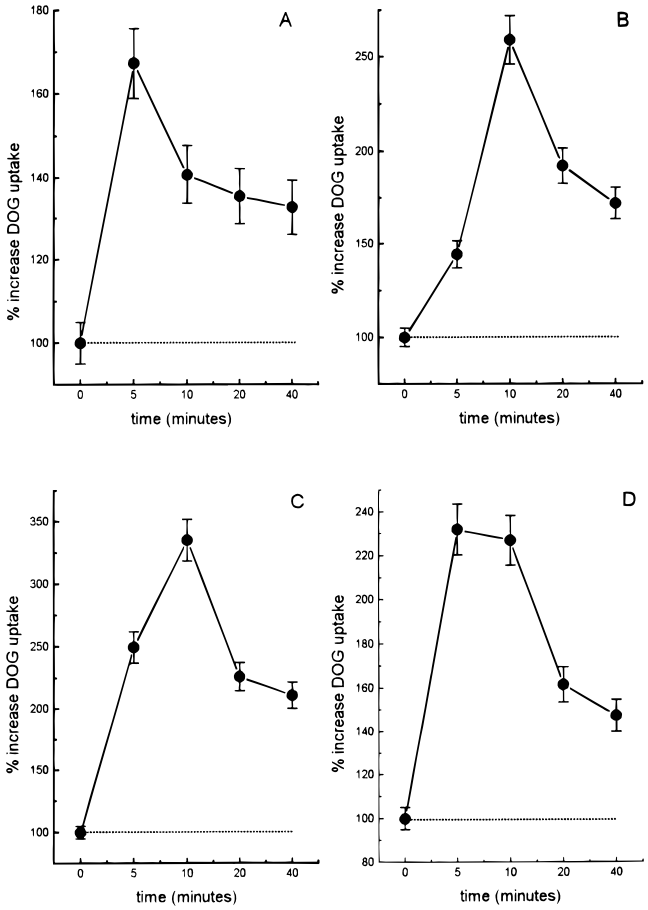


FIGURE 1: Time course of 2-deoxy-D-[2,6-$^3$H]glucose ($^3$H-DOG) uptake: (A) normal human fibroblasts, stimulated with 1.84 nM u-PA; (B) LB6 clone 19 cells, stimulated with 1.84 nM uPA; (C) NCTC human epidermal cells, stimulated with 0.92 nM u-PA; (D) VA-13 transformed human fibroblasts, stimulated with 3.68 nM u-PA. Values are expressed as u-PA-dependent percent glucose transport increase with respect to DOG uptake measured in the presence of 10 $\mu$M cytochalasin B. Since cytochalasin B inhibits only GLUT-dependent glucose transport, the values express the specific glucose transport occurring through the facilitated mechanism. Each value represents the mean ± SD ($n$ = 12). Similar results were obtained when glucose transport was stimulated with ATF (not shown).

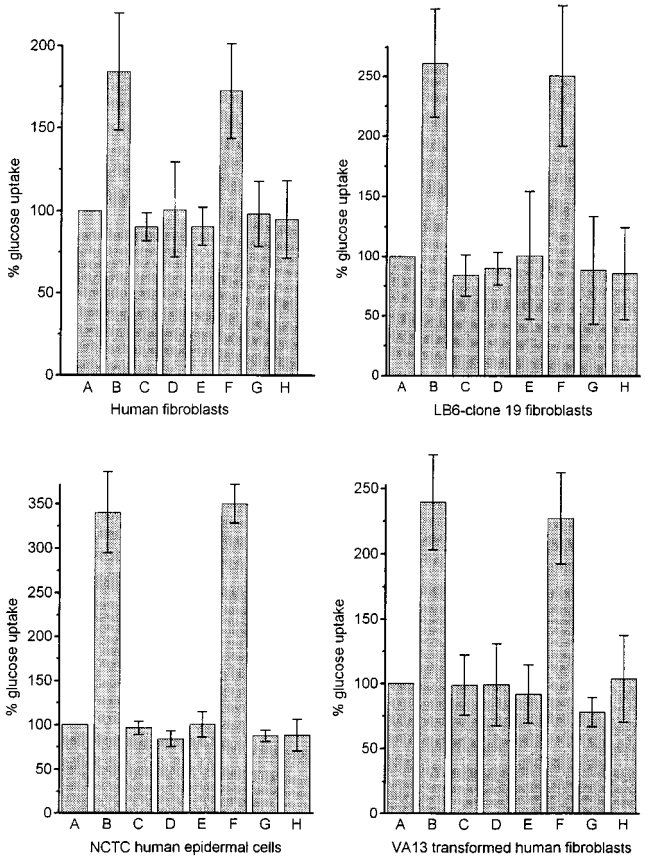


FIGURE 2: Measurement of $^3$H-DOG uptake under the influence of regulative molecules: (A) unstimulated control; (B) stimulation with u-PA; (C) glucose uptake in the presence of 10 $\mu$M cytochalasin B; (D) stimulation with u-PA in the presence of 10 $\mu$M cytochalasin B; (E) glucose uptake in cells incubated overnight with 16 nM PMA; (F) stimulation with u-PA in cells incubated overnight with 16 nM PMA; (G) glucose uptake in the presence of 100 $\mu$M erbstatin; (H) stimulation with u-PA in the presence of 100 $\mu$M erbstatin. Data obtained in the presence of 50 $\mu$g/mL tyrphostin were similar to the data obtained with erbstatin. All the data are referred to the percent variation with respect to unstimulated control, taken as 100%. The u-PA concentrations used were the same as indicated for each cell line in Figure 1. Each value represents the mean ± SD ($n$ = 12).

in a myeloid cell line by a mechanism that involves the activity of tyrosine kinases and protein kinase C (Berridge & Tan, 1995). In order to study whether the u-PA-dependent transporter activation required protein phosphorylation, we investigated 2-DOG uptake in the presence of protein−tyrosine kinase inhibitors and after induction of protein kinase C downregulation with phorbol myristate acetate (PMA) (Figure 2). Tyrphostin and erbstatin, two tyrosine kinase inhibitors, were added to cell monolayers 240 min before 2-DOG uptake, at the concentration of 50 $\mu$g/mL and 100 $\mu$M, respectively. PMA was incubated overnight with cells (at the concentration of 16 nM), before the uptake experiments. In contrast with both tyrosine kinase inhibitors, which strongly inhibited 2-DOG uptake, PKC downregulation was unable to impair hexose transport, and u-PA-stimulated 2-DOG uptake was not significantly affected in PMA-treated cells. These data suggest that protein tyrosine phosphorylation is required to promote the u-PA-dependent facilitative glucose transport, while PMA-activated diacylglycerol-sensitive serine−threonine kinases, whose activation following u-PA/u-PAR interaction has been reported in some cell model systems (Anichini et al., 1994; Busso et al., 1994; He et al., 1991), act probably at later stages downstream the transduction pathway.

*Characterization and Membrane Translocation of the GLUT Isotypes.* Identification of the facilitative glucose transporter molecules involved in the u-PA-dependent increase of 2-DOG uptake was performed by immunoblotting of monolayer cell lysates with monospecific polyclonal rabbit antisera against the various GLUT isoforms. Figure 3 shows the immunoblotting of cell lysates. Each cell line expresses more than one GLUT isoform, allowing us to identify a cell-specific "GLUT mosaic". GLUT2 results the most widespread transporter in our cell lines, while the expression of GLUT1, which is reported to be typical of cultured and transformed cells (Gould & Holman, 1993), is more restricted. GLUT3 is weakly expressed only in transformed fibroblasts, while GLUT4 is absent. It is known that in fat and muscle cells insulin acutely elevates glucose uptake largely by the recruitment to the plasma membrane of preformed glucose transporter proteins (GLUT4 and GLUT1) residing in intracellular organelles (James et al., 1989). Thus, by analogy with the action of insulin on insulin-sensitive tissues, we have investigated whether the increased glucose transport following an acute u-PA treatment (5 min) was

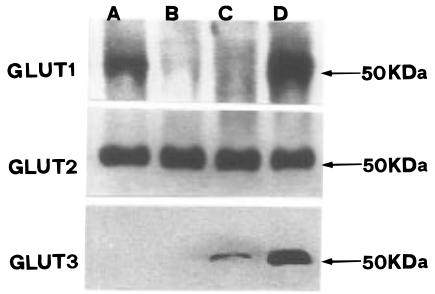


FIGURE 3: Immunoblotting of total cell lysates with rabbit anti-human GLUT antibodies. Polyacrylamide gels (12.5%) of total cell lysates (40 $\mu$g of protein/lane) were blotted onto nitrocellulose and immunoprobed with anti-GLUT antibodies, as described. Secondary antibodies, used at 1:2000 dilution, were peroxidase-conjugated, and the reaction was revealed by ECL. Lanes: (A) LB6 clone 19 fibroblasts; (B) human fibroblasts; (C) NCTC human epidermal cells; (D) VA-13 transformed human fibroblasts. Molecular weights were calculated on the basis of comigration of markers electrophoresed in parallel lanes and stained with Coomassie blue. Anti-GLUT3 polyclonal antibodies differed from others since they were affinity-purified. Immunoblotting with anti-GLUT4 resulted negative (not shown).

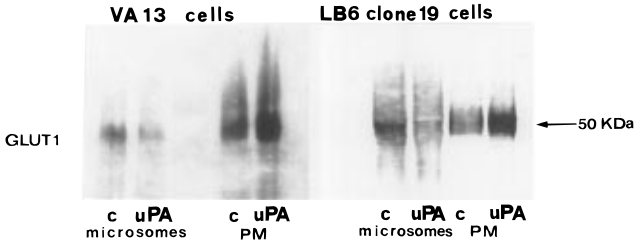


FIGURE 4: Immunoblotting of subcellular fractions with rabbit anti-human GLUT antibodies. Various cell lines were stimulated with different concentrations of u-PA reported in Table 1 and Figure 1. At the time corresponding to maximal increase of DOG uptake (5 min for human fibroblasts, NCTC cells, and VA-13 cells; 10 min for LB6 clone 19 cells), cells were collected, resuspended, homogenized, and fractionated as described. Aliquots of subcellular fractions containing 20 $\mu$g of protein each were subjected to polyacrylamide gel electrophoresis and immunoprobed with anti-GLUT antibodies. Specific bands were revealed by ECL, as described, and subjected to densitometric scanning with an image analyzer. In VA-13 cells 30% of GLUT1 resulted associated to the microsome fraction and 70% to the membrane fraction, under control conditions, while stimulation with ATF or u-PA resulted in a decrease of the microsome fraction (10%) and an increase of the membrane fraction (90%). For LB6 clone 19 cells the percentages of GLUT1 redistribution shifted from 40% in microsomes and 60% in the membrane, under control conditions, to 20% in the microsomes and 80% in the membrane after ATF or u-PA stimulation. No appreciable differences were observed for GLUT2 in both NCTC cells and human fibroblasts (not shown). Abbreviations: c, control unstimulated cells; PM, plasma membranes.

based on a redistribution of glucose transporters by both immunoblotting of subcellular fractions and immunofluorescence of adherent plasma membrane sheets. GLUT2 does not redistribute after u-PA or ATF treatment, while GLUT1 undergoes translocation from the microsomal to the plasma membrane compartment, as shown by immunoblotting of fractionated plasma membranes and microsomes of both LB6 clone 19 cells and transformed human fibroblasts under both control and stimulation conditions (Figure 4). To specifically evaluate the effect of u-PA-ATF on the redistribution of GLUT1, LB6 clone 19 cells grown on glass coverslips were subjected to sonification to prepare plasma membrane sheets. ATF stimulation significantly increased the amount of GLUT1 on the plasma membrane, as assayed by immunofluorescence using anti-GLUT1 antiserum (Figure 5A,B). GLUT2-expressing cells (human fibroblasts in Figure 5C,D) did not show any evidence of translocation, in agreement with the data reported in the literature. Additionally, cytocalasin B pretreatment of cultured cells inhibited the increase of GLUT1 on the plasma membrane in response to u-PAR stimulation (data not shown).

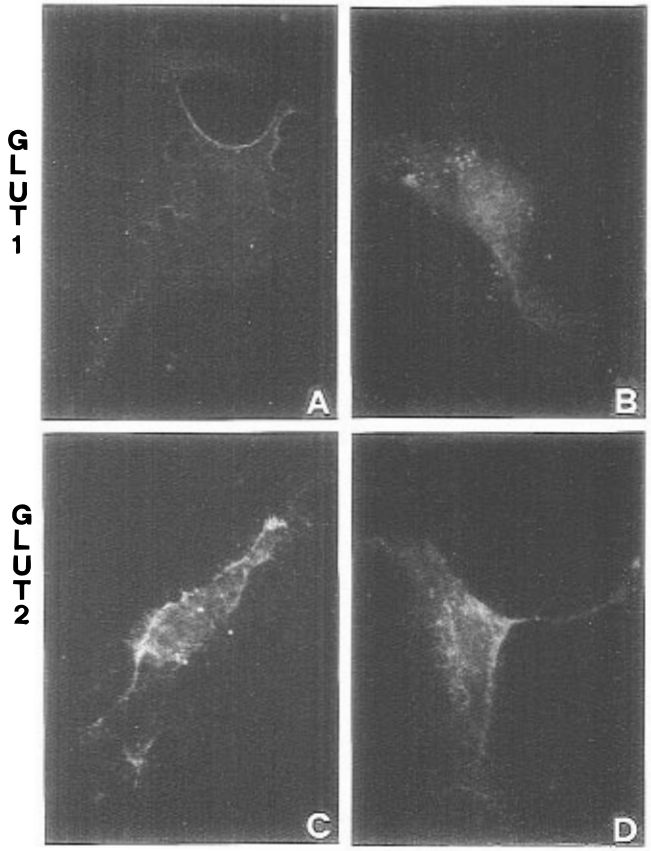


FIGURE 5: Immunofluorescence of plasma membrane sheets to analyze cell surface GLUT1 and GLUT2 expression in response to an acute treatment with u-PA-ATF. Cells were stimulated with u-PA-ATF for 5 min (human fibroblasts) or 10 min (LB6 clone 19 cells). Plasma membrane sheets were then prepared by sonification and subjected to immunofluorescence using anti-GLUT1 (A, B) or anti-GLUT2 (C, D) antisera. Panels: (A) plasma membrane sheets of control unstimulated LB6 clone 19 cells; (B) plasma membrane sheets of LB6 clone 19 cells stimulated with u-PA-ATF for 10 min; (C) plasma membrane sheets of control unstimulated human fibroblasts; (D) plasma membrane sheets of human fibroblasts stimulated with u-PA-ATF for 5 min.

*Dependence of DAG Neosynthesis on u-PA-Induced Glucose Uptake.* Previous results obtained on NCTC 2544 epithelial cells from human epidermis (Del Rosso et al., 1993), LB6 clone 19 mouse fibroblasts transfected with human u-PAR gene, and adult fibroblasts from human skin (Anichini et al., 1994) indicated that DAG production following u-PA/u-PAR interaction derived from *de novo* synthesis from glucose, since the classical pathway of inositol lipid breakdown leading to the formation of inositol phosphates was not activated upon u-PA or u-PA-ATF stimulation. Evaluation of inositol phosphates also in VA-13 cells did not show any modification, as previously shown in other cell lines used in this study (results not shown) (Anichini et al., 1994). In order to evaluate whether DAG neosynthesis depended on the u-PA-dependent increase of glucose uptake, we have measured DAG neosynthesis after treatment of cell monolayers with 10 $\mu$M cytochalasin B, an inhibitor of facilitated glucose transport. Figure 6 shows that cytocha-

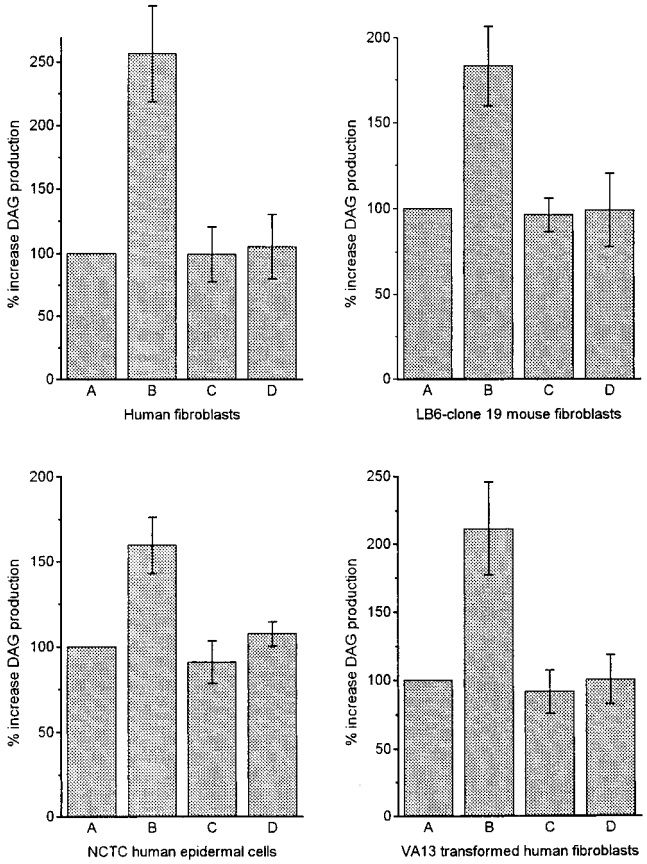


FIGURE 6: Glucose uptake-dependent neosynthesis of diacylglycerol (DAG): (A) DAG neosynthesis under control conditions; (B) DAG neosynthesis after u-PAR stimulation with u-PA; (C) DAG neosynthesis in the presence of 10 $\mu$M cytochalasin B; (D) DAG neosynthesis after u-PAR stimulation with u-PA in the presence of 10 $\mu$M cytochalasin B. u-PA for each cell line was used at the concentrations reported in Figure 1. Data are the mean ± SD ($n$ = 12).

lasin B abolished u-PA-stimulated DAG increase in all cell lines under study, suggesting that the u-PA-dependent intracellular glucose increase is related to downstream metabolic events leading to DAG neosynthesis.

*PKC Activity.* In all experiments performed to examine the effect of u-PAR stimulation on PKC in tissue culture, cells were first grown to confluency and then maintained overnight in serum-free conditions in order to keep the cells in a quiescent state. Stimulation with a concentration of u-PA able to stimulate the maximal increase of glucose transport for 30 min at 37 °C resulted in the translocation of PKC activity in the cytosolic pool, with a concomitant decrease in the membranous fraction. Total PKC activity did not change (Table 2).

*Inhibition of u-PA-Dependent Glucose Uptake, DAG Neosynthesis, and PKC Translocation by Inhibition of u-PAR-Mediated Signaling.* In order to assay the dependence of glucose transport, DAG *de novo* synthesis, and PKC activation on u-PA/u-PAR interaction, we have evaluated each biochemical step following exclusion of u-PAR activity. u-PAR expression was inhibited by anti-u-PAR aODN: semiconfluent cell monolayers were exposed to ODNs for 4 days, on the basis of preliminary experiments indicating a steady reduction of u-PAR number after 3 days of aODN treatment (Quattrone et al., 1995a,b). Since the half-life of DOTAP-combined ODNs in the culture medium was about 48 h, the initial treatment with 10 $\mu$M DOTAP-combined ODNs was followed by a second addition of 5 $\mu$M DOTAP-combined ODNs after 48 h in order to restore the initial concentration. On the fourth day, cells were used for DOG uptake, DAG neosynthesis, and PKC translocation: the results are reported in Table 3. With regard to the effectiveness of aODN and ineffectualness of ssODN in reducing u-PAR number, we refer to previous results from our laboratory on transformed human fibroblasts (Quattrone et al., 1995a,b). It is evident that anti-u-PAR aODNs abolished u-PAR-mediated cell signaling. Abolition of u-PA effect was achieved also in the presence of mAbs against u-PA-ATF (5B4 mAb) and against the u-PA-binding domain of u-PAR (R3 mAb). The experiments were performed in the presence of 1.5 $\mu$g/mL of each mAb and indifferent IgGs were used as negative control (Table 3). Recent data indicate the existence in human platelets of a novel u-PA binding site which has binding kinetics similar to those of u-PAR but which exhibits characteristics of a *bona fide* transmembrane receptor (Jiang et al., 1996). To exclude the possibility that the two types of receptors are coexpressed in our cell lines and that the effects observed may be attributed to such receptors, DOG uptake, DAG neosynthesis, and PKC activation were measured in cells pretreated with bacterial GPI-specific phospholipase C, as described (Anichini et al., 1994) (Table 3). An overview of the data reported in Table 3 suggests that impairment of u-PAR expression with aODN, blockade of u-PA/u-PAR interaction with mAbs, and surface cleaning of u-PAR with phospholipase C abolish u-PA-dependent DOG uptake, DAG *de novo* synthesis, and PKC activation.

Table 2: Subcellular Localization of PKC Activity[a]

| | | LB6 clone 19 | human fiber | NCTC | VA-13 |
|---|---|---|---|---|---|
| control | C[b] | 74.2 ± 6.6* | 61.6 ± 6.3* | 110.9 ± 15 | 60.4 ± 8.2 |
| | PM[c] | 32.8 ± 4.6* | 21.2 ± 4.1* | 6.8 ± 2.2* | 14.3 ± 3.6* |
| +u-PA[d] | C | 98.3 ± 10.1 | 77.9 ± 8.6 | 118.8 ± 14.1 | 71.2 ± 6.9 |
| | PM | 10.5 ± 3.9 | 6.8 ± 1.9 | 1.5 ± 0.5 | 5.1 ± 1.5 |

[a] The activity is expressed as picomoles of phosphate transferred per minute per $1 \times 10^6$ cells. The asterisk indicates significant values ($p < 0.05$, Student's $T$ test for unpaired samples) obtained by comparing the data of PKC activity for C and PM in control cells to the same data in u-PA-stimulated cells. [b] C, cytosol. [c] PM, plasma membrane. [d] The concentrations of u-PA used to stimulate each cell line were as reported in Figure 1. Data reported are the mean ± SD ($n$ = 9).

## DISCUSSION

It is known that growth/chemotactic factors promote cell proliferation and cell movement by activation of signal transduction pathways that result in progression through the cell cycle, differential gene expression, and cell movement. The uptake of simple sugars required for basal cell metabolism and macromolecular synthesis needed for cell growth, proliferation, and migration is thought to follow as a consequence of signal transduction to the nucleus. The demonstration that DAG can be rapidly synthesized *de novo* from glucose in some cell model systems after stimulation with ligands or by increased extracellular glucose concentration (Farese et al., 1986; Peter-Riesch et al., 1988; Rossi et al., 1991; Lee et al., 1989a,b) has led to the hypothesis that, owing to the function of DAG as a second messenger, this signaling pathway could be a mechanism by which some surface recognition systems transduce external signals into cellular functions. In previous papers (Del Rosso et al., 1993; Anichini et al., 1994) we have shown that *de novo* synthesis

Table 3: Inhibition of DOG Uptake, DAG Neosynthesis, and PKC Translocation[a]

| | | human fibr | LB6 clone 19 | NCTC | VA-13 |
|---|---|---|---|---|---|
| DOG[b] | control | 100 | 100 | 100 | 100 |
| | u-PA[c] | 167* ± 12 | 258* ± 14 | 334* ± 21 | 230* ± 15 |
| | u-PA + ssODN[d] | 153* ± 21 | 222* ± 35 | 283* ± 65 | 186* ± 51 |
| | u-PA + aODN[e] | 96 ± 13 | 81 ± 18 | 93 ± 21 | 88 ± 28 |
| | i-IgG[f] | 105 ± 11 | 103 ± 12 | 99 ± 10 | 106 ± 8 |
| | u-PA + 5B4[g] | 106 ± 15 | 98 ± 10 | 106 ± 7 | 92 ± 9 |
| | u-PA + R3[h] | 101 ± 18 | 103 ± 14 | 95 ± 11 | 106 ± 21 |
| | u-PA + GPI-PLC[i] | 103 ± 13 | 101 ± 17 | 97 ± 9 | 99 ± 12 |
| DAG[b] | control | 100 | 100 | 100 | 100 |
| | u-PA | 252* ± 45 | 180* ± 25 | 161* ± 17 | 210* ± 35 |
| | u-PA + ssODN | 236* ± 61 | 158* ± 30 | 151* ± 18 | 203* ± 41 |
| | u-PA + aODN | 83 ± 18 | 91 ± 26 | 88 ± 31 | 98 ± 19 |
| | i-IgG | 103 ± 15 | 96 ± 8 | 99 ± 12 | 108 ± 16 |
| | u-PA + 5B4 | 97 ± 13 | 86 ± 11 | 103 ± 14 | 95 ± 16 |
| | u-PA + R3 | 87 ± 8 | 95 ± 12 | 93 ± 13 | 101 ± 14 |
| | u-PA + GPI-PLC | 108 ± 11 | 113 ± 10 | 115 ± 9 | 106 ± 7 |
| PKC[j] | control | 100 | 100 | 100 | 100 |
| | u-PA | 31* ± 12 | 32* ± 9 | 22* ± 7 | 36* ± 10 |
| | u-PA + ssODN | 38* ± 10 | 33* ± 11 | 26* ± 9 | 38* ± 9 |
| | u-PA + a ODN | 95 ± 12 | 103 ± 14 | 99 ± 15 | 110 ± 13 |
| | i-IgG | nd[k] | nd | nd | 96 ± 18 |
| | u-PA + 5B4 | nd | nd | nd | 108 ± 17 |
| | u-PA + R3 | nd | nd | nd | 91 ± 16 |
| | u-PA + GPI-PLC | 106 ± 12 | 93 ± 15 | 97 ± 9 | 106 ± 15 |

[a] The asterisk indicates values significantly different ($p < 0.05$, Student's $T$ test for unpaired samples) from the control. [b] Values are expressed as percent of control value taken as 100% and represent the mean ± SD ($n = 4$, unless otherwise stated). [c] u-PA concentration for each cell line was as reported in Figure 1. [d] ssODN, scrambled sequence oligodeoxyribonucleotides. [e] aODN, antisense oligodeoxyribonucleotides. [f] i-IgG, indifferent mouse IgG. [g] Mouse mAb 5B4, anti-amino-terminal fragment of human u-PA. [h] Mouse mAb R3, anti-u-PA-binding sequence of u-PAR. [i] Glycosylphosphatidylinositol-specific phospholipase C from *Bacillus cereus*. The enzyme was used at the final concentration of 0.4 μg/mL in the culture medium, as described (Del Rosso et al., 1992). [j] The values of PKC indicate the percent activity remaining at the cell membrane after stimulation with u-PA under the experimental conditions described. [k] nd, not determined.

from glucose was the only source of DAG production following u-PA interaction with membrane u-PAR. In this study we show that DAG *de novo* synthesis following u-PAR stimulation is regulated by glucose uptake by a mechanism involving tyrosine kinases and activation or translocation of GLUTs at the cell membrane. Kinetic analysis of glucose transport following uPAR stimulation with u-PA or u-PA-ATF at concentrations known to trigger chemotaxis showed that glucose uptake reaches a peak in 5−10 min depending on the cell line. Both GLUT1- and GLUT2-expressing cell lines actively augment glucose uptake in response to u-PAR stimulation. While GLUT1 undergoes redistribution from microsomes to the plasma membrane, as shown by both immunoblotting and immunofluorescence, GLUT2 does not show any translocation, in agreement with previous data (Brant et al., 1994). The molecular background of the increased GLUT activity is actually unknown: the transport of glucose across the lipid bilayer by facilitative transporters is believed to occur by a mechanism in which the transporters alternate between two conformational states with the substrate-binding site facing either the extracellular of cytoplasmic side of the membrane. Such conformational states, as well as GLUT4 and GLUT1 redistribution, have been suggested to be regulated by protein phosphorylation (Berridge & Tan, 1995; Clarke et al., 1994; Nishimura & Simpson, 1994). Our data show that inhibition of tyrosine phosphorylation by tyrphostin and erbstatin inhibits the increase of glucose uptake, which is unaffected by PKC downregulation. Taken together, our data indicate the following properties of u-PAR-dependent stimulation of glucose uptake, which are similar for all cell lines examined: (1) both u-PA and u-PA-ATF stimulate glucose uptake; (2) glucose uptake is of the facilitative type, since it is inhibited by cytocalasin B; (3) such an increase occurs by redistribution of GLUT1 or by activation of GLUT2; (4) it is dependent on tyrosine phosphorylation and independent of PKC activation; (5) intracellular glucose increase is paralleled by *de novo* synthesis of DAG, which is inhibited by impairment of active glucose uptake by cytocalasin B; (6) u-PAR-dependent PKC activation, resulting in the redistribution of PKC activity between the cytoplasm and the cell membrane, is inhibited by impairment of glucose uptake. On the basis of our results and of previous data obtained in both our and other laboratories, we hypothesize the following sequence of events in the chemotactic interaction between u-PAR and u-PA/u-PA-ATF: the ligand/receptor interaction is followed by fast activation of a tyrosine kinase (probably of the *src* family) (Resnati et al., 1996; Bohuslav et al., 1995), which phosphorylates substrates involved with translocation of GLUT1 from the microsome to the plasma membrane compartment or with direct activation of GLUT2 on the surface membrane; the increase in glucose uptake, which in some cell lines can augment 3-fold the intracellular glucose concentration, stimulates DAG neosynthesis, which in turn activates PKC. It is likely that PKC activation leads to phosphorylation and assembly of proteins that are essential for cells to migrate and invade, such as cytokeratin 18 and 8 (Busso et al., 1994; Omary et al., 1992). It is noteworthy that u-PAR-dependent PKC activation results in the association of the enzyme activity with the cytosol fraction. This observation is probably related with the formation of the proteolytically activated form of PKC (protein kinase M), whose generation has originally been reported to occur during activation of human neutrophils (Pontremoli et al., 1990). There is evidence that PKM associates with and phosphorylates cytokeratin polypeptides 8 and 18 (Busso et al., 1994; Omary et al., 1992). The functional role of intermediate filament protein phosphorylation is not well understood, but phosphorylation of vimentin, desmin, neurofilaments, nuclear lamins, and cytokeratins appears to be important in regulating

the assembly of these filaments during mitosis and cell movement (Inagaki et al., 1987; Gonda et al., 1990; Busso et al., 1994). Another opened question in the mechanism of transduction through u-PAR is related with its GPI anchor as the only structure connecting u-PAR to the outer leaflet of the plasma membrane (Ploug et al., 1991). Such membrane anchorage presumably cannot directly interact with other transducing molecules, such as protein tyrosine kinases (PTKs) or G-proteins, which are confined to the inner leaflet of the plasma membrane. Therefore, the existence of an adaptor molecule has been suggested, able to couple extracellular contact with intracellular transducing mechanisms. Some possible candidates have been described, such as caveolin (Lisanti et al., 1994; Stahl & Muller, 1995) and $\beta$2-integrins LFA-1 and CR3 (Bohuslav et al., 1995); however, only indirect evidence of interaction has been provided, such as colocalization by confocal microscopy, antibody-induced cocapping, and immune precipitation. We believe that, whatever the adapter, such a molecule has to account for fast stimulation of glucose influx and *de novo* synthesis of DAG without stimulation of phosphatidylinositol hydrolysis. Since u-PAR belongs to the family of GPI-anchored cell surface receptors and it is localized in caveolae in some cell lines (Stahl & Muller, 1995), as well as GLUT4 (Scherer et al., 1994), we have addressed the intriguing possibility that glucose transporters may associate physically with u-PAR: Triton-insoluble complexes of our cell lines were immunoprobed with anti-caveolin, anti-u-PAR, and anti-GLUTs antibodies. The results (not shown) were negative, indicating that the three molecules behaved as independent variables (manuscript in preparation).

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