Glucose regulates protein catabolism in *ras*-transformed fibroblasts through a lysosomal-dependent proteolytic pathway

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Transformed cells are exposed to heterogeneous micro-environments, including low D-glucose (Glc) concentrations inside tumours. The regulation of protein turnover is commonly impaired in many types of transformed cells, but the role of Glc in this regulation is unknown. In the present study we demonstrate that Glc controls protein turnover in *ras*-transformed fibroblasts (KBALB). The regulation by Glc of protein breakdown was correlated with modifications in the levels of lysosomal cathepsins B, L and D, while autophagic sequestration and non-lysosomal proteolytic systems (m- and μ -calpains and the zeta-subunit of the proteasome) remained unaffected. Lactacystin, a selective inhibitor of the proteasome, depressed proteolysis, but did not prevent its regulation by Glc. The sole inhibition of the

cysteine endopeptidases (cathepsins B and L, and calpains) by E-64d [(2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester] was also not sufficient to alter the effect of Glc on proteolysis. The Glc-dependent increase in proteolysis was, however, prevented after optimal inhibition of lysosomal cysteine and aspartic endopeptidases by methylamine. We conclude that, in transformed cells, Glc plays a critical role in the regulation of protein turnover and that the lysosomal proteolytic capacity is mainly responsible for the control of intracellular proteolysis by Glc.

Key words: cathepsin, glycolysis, proteasome, proteolysis, tumour cell.

INTRODUCTION

Extensive nutritional and metabolic alterations frequently occur in cancer patients. Loss of host adipose tissue and muscle protein, together with disturbances in carbohydrate metabolism, commonly accompanies tumour development [1–4]. Weight loss is in fact a prognostic indicator of survival time in a variety of human malignancies, although the benefits of nutrition support regarding host survival remain controversial [5,6].

The reasons for depletion of host tissue in the tumour-bearing state remain speculative, but may partly be linked to the metabolic properties of the tumour cells. Tumour and transformed cells are known to exhibit profound metabolic deviations, including aerobic glycolysis, glutaminolysis, Crabtree effect, and enhanced nucleic acid and lipid syntheses [7]. The metabolic deviations and consequent demand for nutriment by cancer cells are then not only important for tumour growth, but may in turn participate in the development of the cachectic conditions [8]. Among the metabolic deviations described in tumour cells, an increased glycolytic capacity is one of their most characteristic phenotypes [7,9]. This places a high metabolic demand for D-glucose (Glc) on the host, and, despite increased liver gluconeogenesis, low blood Glc concentrations and decreased Glc utilization by host organs are often associated with cancer cachexia [2,4,10].

In addition to metabolic deviations in tumour-bearing organisms, the intra-tumour distribution of growth factors and of other important substrates, such as Glc, is heterogeneous [11]. Indeed, deficiency of neovascularization is evident in metastatic tumours, even in well-vascularized tissues [12]. In addition, cancer cells in tumours exist as highly packed multicellular aggregates with reduced diffusivity of substances in the interstitial fluid [13]. Gradients of nutriment, metabolites and growth factors therefore exist inside tumours, and cancer cells are

exposed to heterogeneous microenvironments, including low Glc concentrations [11–14].

A selective advantage of tumour cells is their capacity to adapt their metabolism to diverse nutritional conditions [7,9]. A well-known example is their ability to use, according to the environmental conditions, aerobic glycolysis or glutaminolysis as a major source of energy [7,15]. Other important metabolic pathways are affected by growth-factor concentration or nutrient availability. Studies of protein metabolism in transformed cells revealed that protein synthesis is stimulated, and protein breakdown reduced, by serum, but that both processes present a smaller response to growth factors when compared with normal cultured cells [16,17]. Nutrients also play an important role in the control of protein metabolism in tumour cells, and evidence has been provided showing that amino acid supplementation of patients with cancer stimulates protein synthesis of tumour cells [18,19]. The dependence of protein synthesis on cytoplasmic glycolysis was also reported in ascites-tumour cells [20]. However, despite evidences of cancer-associated disturbances in carbohydrate metabolism [2,4,10] and of Glc gradients inside tumours [12–14], few studies have assessed the potential role of Glc on protein turnover in tumour cells. A better understanding of the mechanisms that control proteolysis in transformed cells appears critical, as recent data emphasize that reduced lysosomal autophagic proteolysis favours tumorigenesis [21,22].

In the present study we investigated intracellular protein breakdown in fibroblasts (KBALB) transformed by *ras*, an oncogene which has a major function in carcinogenesis [23] and is implicated in a variety of tumour types [24]. We demonstrate that Glc plays a critical role in the control of intracellular protein turnover in KBALB fibroblasts. In particular, we establish that the Glc-dependent control of protein breakdown is selectively associated with, and dependent on, variations of lysosomal

Abbreviations used: Glc, D-glucose; KBALB, *ras*-transformed (Kirsten-sarcoma-virus-transformed) fibroblast; DMEM, Dulbecco's modified Eagle's medium; Z-, benzoyloxycarbonyl; -NMEc, 7-(4-methyl)coumarylamide; E-64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester.

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endopeptidases. At the low Glc concentrations found in tumour micro-regions, lysosomal proteolysis strongly decreases, and may provide a selective advantage to transformed cells.

MATERIALS AND METHODS

Reagents

Cell lines were purchased from the American Type Culture Collection (Rockville, MD, U.S.A., but now at Manassas, VA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), foetal-calf serum and gentamycin were from Gibco BRL (Cergy Pontoise, France). Benzyloxycarbonyl (Z)-Arg-Arg 7-(4-methyl)-coumarylamide (-NMec), Z-Phe-Arg-NMec and Z-Phe-Phe-CHN₂ were obtained from Bachem (Voisins-le-Bretonneux, France). The Aurora Western detection kit and alkaline phosphatase-conjugated IgG were from ICN Biomedicals (Orsay, France). L-[³⁵S]Methionine was obtained from Amersham Pharmacia Biotech (Orsay, France) and the ATP bioluminescence Assay kit HS II from Boehringer Mannheim (Roche Diagnostics, Meylan, France). PVDF membranes, Glucose kit, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64d), concanamycin-A and all other chemicals (analytical grade) were purchased from Sigma (Sigma-Aldrich, L'Isle-d'Abeau Chesnes, France).

Cell culture

Murine BALB/3T3 clone A31 cells and BALB/3T3 cells transformed with the Kirsten sarcoma virus (KBALB) were propagated according to the supplier's recommendations. The transforming activity of Kirsten virus is attributed to the expression of K-ras which is a constitutively active mutated form of cellular Ras. Glc treatment was initiated by incubating confluent cells (7 days and 3 days after plating for BALB/3T3 and KBALB respectively) in DMEM supplemented with 50 µg/ml gentamycin, 3.7 mg/ml NaHCO₃ and 10% (v/v) foetal-calf serum and containing 4 mM glutamine, 1.3 mM pyruvate and either 1 or 4 g/l Glc (DMEM1 or DMEM4 respectively). Cells were fed with fresh medium daily.

Cathepsin activities

KBALB cells were washed with PBS, pH 7.4, detached with a cell scraper and disrupted by successive aspirations through a 26-gauge needle into 2 ml of 0.25 M saccharose/25 mM Hepes/2 mM EDTA, pH 7.4. Cellular homogenates were centrifuged at 2000 g for 2 min, the postnuclear supernatants were centrifuged at 70000 g for 15 min to discard cytosolic inhibitors, and the pellets were used to assay lysosomal-endosomal endopeptidases. Cysteine-endopeptidase activities were measured in 0.1 M acetate buffer, pH 5.5, containing 1 mM EDTA and 5 mM dithiothreitol and using 50 µM Z-Arg-Arg-NMec or 10 µM Z-Phe-Arg-NMec as substrates [25]. Z-Arg-Arg-NMec is a specific substrate for cathepsin B [26]. Under our assay conditions, 90% of Z-Phe-Arg-NMec-hydrolysing activity was inhibited by neutral pH or by Z-Phe-Phe-CHN₂ (2 µM), a specific inhibitor of cathepsin L, and therefore Z-Phe-Arg-NMec hydrolysis essentially reflected cathepsin L activity [27]. For cathepsin B and cathepsin L, one unit of activity is defined as the release of 1 µmol of fluorescent product/min. Cathepsin D aspartic endopeptidase activity was assayed in 0.2 M formate buffer, pH 3.5, using 3% (w/v) bovine haemoglobin as substrate, the reaction was stopped by 5% (w/v, final) trichloroacetic acid and the peptides liberated in the supernatant were measured by their absorbance at 280 nm.

Immunoblotting

Cells were lysed in sample buffer for SDS/PAGE, and aliquots (20 µg of protein) were electrophoresed under reducing conditions on SDS/10% PAGE. The separated proteins were electrotransferred to PVDF membranes in 48 mM Tris/30 mM glycine, pH 8.3, containing 20% (v/v) methanol. The membranes were developed with an enhanced-chemiluminescence Western detection system using 0.2% (w/v) reconstituted dried milk and 0.1% (v/v) Triton X-100 as blocking agents. Primary antibodies were rabbit IgG raised against mouse cathepsin L [28] or mouse cathepsin B [29], or mouse IgG anti-(proteasome subunit zeta) antibody (MCP196) [30]. The antipeptide antibody to calpains was produced by immunization of a rabbit with synthetic peptides derived from m-, µ- and p94-calpains, and conjugated to keyhole-limpet (*Diodora aspera*) haemocyanin (A. Ouali and Y. Benyamin, personal communication). The secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit IgG or duck anti-mouse IgG.

Protein turnover

For protein-breakdown measurements, confluent KBALB or BALB/3T3 cells (in 6 cm-diameter Petri dishes) were labelled for 15 h with L-[³⁵S]methionine (10 µCi/ml) in 2 ml of DMEM1 or DMEM4. Chases were performed with normal or conditioned medium, supplemented with 2 mM unlabelled L-methionine (chase medium). Conditioned medium was DMEM1 or DMEM4 incubated for 15 h with confluent KBALB. After labelling, the cells were washed three times with chase medium and left for 3 h in this same medium to permit breakdown of short-lived proteins. The degradation period was started (zero time) by replacing the medium with 2 ml of chase medium, and 20 µl aliquots of extracellular medium were collected at the indicated times. Extracellular proteins were precipitated for 15 min at 4 °C with trichloroacetic acid [12% (w/v) final concn.] in the presence of 12 µg of BSA and centrifuged for 10 min at 12000 g. The pellet was solubilized in 0.5 M NaOH/0.1% (v/v) Triton X-100 to determine acid-insoluble radioactivity in the medium. The supernatant was taken for radioactivity measurement of acid-soluble amino acids produced by protein degradation (*S_t*). At the end of the degradation period, cells were fixed at 4 °C with 0.6 ml of 10% (w/v) trichloroacetic acid and solubilized in 0.5 M NaOH/0.1% (v/v) Triton X-100 to measure the residual radioactivity of cell proteins. The rate of protein degradation (*k_d*) was evaluated by the equation describing a first-order decay [31]:

$$A_t = A_0 \exp(-k_d t)$$

where *A₀* is total radioactivity incorporated in cell proteins at zero time and *A_t* (= *A₀* - *S_t*) is the radioactivity remaining at time *t* (*S_t* is the radioactivity of acid-soluble amino acids). Measurements of protein-synthesis rates were conducted as described previously [32].

Lactate dehydrogenase sequestration

To measure lactate dehydrogenase sequestration, KBALB cells incubated in DMEM1 or DMEM4 were treated for 0–5 h with vehicle alone [0.1% (v/v) DMSO; control] or with 1 µM concanamycin-A, a selective inhibitor of vacuolar H⁺-ATPase, which impairs cathepsin activities and trafficking to lysosomes [33]. KBALB were extracted and lactate dehydrogenase activity was measured in cellular homogenate and in 70000 g pellet by monitoring the oxidation of NADH with pyruvate as substrate at 340 nm [34].

Other procedures

Extracellular Glc was measured using a Glucose kit, protein concentrations were determined according to the bicinchoninic acid ('BCA') method [35], and cellular ATP was assayed with the ATP bioluminescence assay kit HS II.

RESULTS

Glc increases protein-breakdown and -synthesis rates in KBALB fibroblasts

Preliminary experiments performed with confluent KBALB fibroblasts revealed that the degradation of long-lived proteins differed when the cells were incubated in medium containing 1 g/l (DMEM1) or 4 g/l Glc (DMEM4), despite the presence of 10% (v/v) serum and of amino acids. Intracellular protein degradation was decreased when [35 S]methionine-labelled KBALB cells were incubated in DMEM1, but only after 10 h of chase (Figure 1A). In contrast with what was observed with KBALB cells, no modification of protein breakdown could be seen when similar experiments were performed with non-transformed BALB/3T3 fibroblasts (Figure 1C).

The Glc-dependent modification of proteolysis in KBALB cells was reproduced without delay when chases were performed with conditioned media (Figure 1B). This indicated that modifications, important for proteolysis, occurred in the extracellular medium during 10 h of incubation. Further analyses of the medium revealed high rates of Glc consumption by KBALB cells (0.73 ± 0.06 and 0.78 ± 0.04 μ g/h per mg of protein in DMEM1 and DMEM4 respectively; $n = 6$ in each group). Similar rates of Glc consumption have been reported for colon-carcinoma [14] or breast-cancer cells [36]. These high rates of Glc consumption by KBALB cells generated a low Glc concentration (0.05–0.1 mM) and a reduced proteolysis after 10 h in DMEM1. In contrast, when DMEM4 was used, Glc was maintained at > 1 g/l and KBALB cells maintained high rates of proteolysis. High rates of proteolysis were not due to an excess of Glc and occurred when Glc was not limiting: in DMEM4, but also at early times (< 10 h) in DMEM1 (Figure 1A), or when fresh DMEM1 was regularly provided during the chase (results not shown).

KBALB cells are classically grown in DMEM1, and no major differences other than Glc concentration could be detected between conditioned DMEM1 and conditioned DMEM4, nor was there evidence for alterations in cell viability. In particular, extracellular acid-insoluble radioactivity remained at a low level under all conditions (Figure 1), and lactate dehydrogenase was not measurable in the medium. Cellular ATP did not reveal any difference (2.4 ± 0.4 and 2.0 ± 0.3 nmol/mg of protein respectively; $n = 9$) whether KBALB cells were incubated in DMEM1 or DMEM4, probably because glutamine (4 mM in DMEM) is a major source of energy for transformed cells [37].

In KBALB cells, the Glc-dependent increase in proteolysis did not result in a net loss of cellular proteins (3.7 ± 0.8 and 4.6 ± 1.1 mg/dish after 2 days in DMEM1 and DMEM4 respectively; $n = 6$). In fact, measurements of incorporation of L-[35 S]methionine/L-[35 S]cysteine in confluent KBALB cells also revealed higher rates of intracellular protein synthesis in DMEM4 than in DMEM1 (1680 ± 170 and 980 ± 80 d.p.m./h per μ g of protein respectively; $n = 3$). These data emphasized that Glc controls protein turnover in confluent KBALB cells. Because gradients of Glc concentrations are also encountered inside tumours [11–14], the present model was thereafter used to investigate the mechanisms according to which Glc availability modulates protein breakdown in *ras*-transformed cells.

Glc preferentially increases endosomal–lysosomal endopeptidases, but not other proteolytic systems, in *ras*-transformed fibroblasts

Several proteolytic systems have been implicated in intracellular protein breakdown: cathepsin-dependent lysosomal–endosomal system [38], ubiquitin–proteasome-dependent system [39] and Ca^{2+} -dependent calpains [40]. In a previous study [27], we found that Glc strongly increases active forms of cathepsins B, L and D in endosomes–lysosomes of confluent KBALB cells. We, however, did not assess whether Glc also controls the expression of intracellular proteolytic systems other than cathepsins. To test this hypothesis, KBALB were incubated for 20 h in DMEM1 or DMEM4 in order to modify protein turnover, and total cellular extracts were prepared and analysed by Western blotting (Figure 2). Antibodies immunospecific for the constitutive α -type zeta-subunit of the proteasome [30] recognize a single 28 kDa band.

Figure 1 Glucose (Glc) stimulates proteolysis rates in KBALB cells

Confluent BALB/3T3 (C) or KBALB (A and B) fibroblasts were labelled for 15 h with 10 μ l/ml L-[35 S]methionine in medium containing 1 g/l Glc (DMEM1, open symbols) or 4 g/l Glc (DMEM4, closed symbols). Chases were performed with normal (A and C) or conditioned (B) DMEM1 or DMEM4, supplemented with 2 mM unlabelled methionine. Conditioned media (cDMEM) were from parallel non-radioactive cultures. Protein degradation (circles) and extracellular acid-insoluble radioactivity (triangles; protein secretion and/or cell death) were estimated as described in the Materials and methods section. Values are means $\pm$ S.D. ($n = 3$).

Figure 2 Glc selectively induces cathepsins, but not other proteolytic systems, in KBALB cells

Confluent KBALB cells were incubated for 20 h in DMEM1 or DMEM4 in order to modify rates of protein degradation. Similar incubations were performed with non-transformed BALB/3T3 fibroblasts. Cell lysates (20 µg of protein) were prepared, subjected to SDS/PAGE and immunoblotted with anti-(proteasome zeta-subunit), anti-calpains, anti-(cathepsin L) or anti-(cathepsin B) antibodies.

Immunoblotting with the anti-calpains antibody labelled the large (80 kDa) and small subunits (30 kDa) of μ - and m-calpains. Neither the 30 or the 80 kDa calpain subunits, nor the proteasome zeta-subunit, revealed major variations when KBALB were incubated in DMEM4 instead of DMEM1. Immunoblotting with the anti-calpains antibody revealed that Glc treatment of KBALB induced a 94 kDa band. This 94 kDa band may correspond to p94-calpain, whose enzymic properties have not yet been demonstrated [41]. In contrast with proteasome zeta-subunit and μ - and m-calpains, active forms of cathepsins B (32 kDa) and L (29 kDa and 21 kDa) were strongly increased by Glc in KBALB cells. Increased concentrations of active cathepsins were also associated with higher levels of lysosomal–endosomal endopeptidase activities (see Figure 3B).

In non-transformed BALB/3T3 fibroblasts, similar experimental procedures did not alter protein turnover, and also did

not modify cathepsin B and cathepsin L levels (Figure 2). In BALB/3T3 cells, Glc also slightly increased the 80 kDa calpain subunit, while the zeta-subunit of the proteasome was hardly detectable, in agreement with the previously reported high expression of proteasomes in malignant tumour cells [42].

As a whole, these observations revealed that, in *ras*-transformed cells, but not in non-transformed cells, Glc selectively controls the expression of active forms of lysosomal–endosomal endopeptidases, without affecting to similar extents the non-lysosomal proteolytic systems.

Glc induction of protein-breakdown rates is not prevented by lactacystin or E-64d

Our observation that Glc increases active cathepsins and in parallel stimulates breakdown rates of intracellular proteins did not mean, however, that the two events were necessarily related. To specify the relative role of the different proteolytic systems in the Glc-induced proteolysis, various inhibitors were then assessed.

The *Streptomyces* metabolite lactacystin is spontaneously converted in the extracellular medium into cell-permeant *clasto*-lactacystin β -lactone, which is a specific inhibitor of proteasome activity [43,44]. In particular, incubation of KBALB fibroblasts with lactacystin did not affect lysosomal cathepsin activities (Figure 3). Lactacystin strongly inhibited (by 40–45%), but to a similar extent, intracellular protein breakdown in KBALB incubated in DMEM1 or DMEM4. In particular, lactacystin failed to block Glc effect on proteolysis (Figure 3A). Because Glc control of intracellular proteolysis in KBALB fibroblasts was neither associated with detectable variations in 20 S-proteasome zeta-subunit, nor prevented by lactacystin, proteasome-dependent proteolysis appeared unlikely to play a major role in the control by Glc of protein breakdown in KBALB cells.

The cell-permeant epoxysuccinyl derivative E-64d is known to selectively inhibit cysteine endopeptidases (including cathepsins B and L, and calpains) [45]. In KBALB cells, E-64d rapidly and considerably inhibited cathepsin B and cathepsin L activities (Figure 3B). E-64d also reduced (by 25–30%) proteolysis rates in DMEM1 and DMEM4. However, like lactacystin, E-64d did not offset the control by Glc of protein breakdown (Figure 3A). These data indicated that blocking the cysteine endopeptidases

Figure 3 Inhibition of protein catabolism in KBALB cells by lactacystin or E-64d

(A) Confluent KBALB cells were incubated in DMEM1 (open symbols) or DMEM4 (closed symbols) and L -[35 S]methionine-labelled as in Figure 1. Chases were performed with conditioned DMEM1 or DMEM4, supplemented with 2 mM unlabelled L -methionine, and in the presence or not of 10 µM lactacystin or 10 µM E-64d. Protein degraded (circles) and extracellular acid-insoluble radioactivity (triangles) were determined at the indicated times, as described in the Materials and methods section. (B) Similar incubations were performed in DMEM1 (stippled bars) or DMEM4 (black bars), but without radioactive labelling to measure lysosomal–endosomal activities of cathepsin B and cathepsin L. Values are means $\pm$ S.D. ($n = 4$). * $P < 0.01$ indicates statistically significant differences from the group incubated in DMEM1. Abbreviation: U, units.

Figure 4 Methylamine prevents Glc-dependent induction of cathepsin activities and protein breakdown in KBALB fibroblasts

KBALB cells were incubated in DMEM1 (open circles) or DMEM4 (closed circles) and L-[35 S]methionine-labelled as described in Figure 3. Chases were performed for 15 h with conditioned DMEM1 or DMEM4, supplemented with 2 mM unlabelled L-methionine and the indicated concentrations of methylamine. Rates of intracellular protein degradation (k_d) were estimated as described in Materials and methods section. Similar incubations were performed without radioactive labelling to measure lysosomal–endosomal activities of cathepsin B (CB), cathepsin L (CL) and cathepsin D (CD). Values shown represent means $\pm$ S.D. ($n = 4$). Further abbreviations: OD₂₈₀, A_{280} ; Prot, of protein; U, units.

(lysosomal cathepsins B and L, and calpains) is not sufficient to prevent Glc's effect on proteolysis in KBALB cells.

Inhibition of lysosomal endopeptidases prevents Glc induction of proteolysis

Although E-64d inhibits to a large extent cellular cysteine endopeptidases, it nevertheless does not totally block endosomal/lysosomal proteolysis. Indeed E-64d does not affect aspartic endopeptidases (cathepsin D), and the persistence of one type of lysosomal endopeptidase may suffice for lysosomal proteolysis to proceed. Pepstatin is a selective inhibitor of aspartic endopeptidases *in vitro*, but is not readily membrane-permeant [46], and at 1–10 μ M concentration was unable to inhibit *ex vivo* intracellular cathepsin D activity even after 24 h treatment of KBALB cells (results not shown).

Weak-base amines are classically used to inhibit lysosomal proteolysis [47]. By accumulating in acidic vesicles, they increase the luminal pH and thereby inactivate acidic endopeptidases (cathepsins B, L and D). Only partial inhibition of cathepsin activities could be observed, even after prolonged incubation of KBALB in the presence of 10 mM methylamine (Figure 4). Partial resistance of transformed cells to neutralization by weak-

Figure 5 Glc does not affect autophagic sequestration in KBALB fibroblasts

KBALB cells pre-incubated for 15 h in DMEM1 (open symbols) or DMEM4 (closed symbols) were treated with vehicle alone (squares) or with 1 μ M concanamycin-A (triangles) to inhibit lysosomal proteolysis and delivery of autophagosomes to lysosomes. Autophagic sequestration of lactate dehydrogenase was measured at the indicated times. Values are means $\pm$ S.D. ($n = 3$ –6). * $P < 0.05$, ** $P < 0.01$ indicate statistically significant differences from the control.

base amines was previously noted [48]. Accordingly, 10 mM methylamine only partially inhibited protein breakdown in KBALB cells. Dose-dependent studies were then carried out to improve the inhibition of endosomal–lysosomal endopeptidases in KBALB cells. As shown in Figure 4, 40 mM methylamine was found to rapidly inhibit cathepsin D, B and L activities in KBALB cells. Furthermore, under these conditions, inhibition of vesicular endopeptidases was found to totally prevent the Glc effect on rates of protein degradation in KBALB fibroblasts.

Higher levels of active cathepsins may not be the only requirement for increased lysosomal-dependent protein breakdown. Protein substrates also need to be efficiently targeted to lysosomes. Additional experiments were then performed to investigate whether Glc also affects the sequestration of a substrate protein within lysosomes. As indicated in Figure 5, sequestration of a cytosolic enzyme, lactate dehydrogenase, only slightly and not significantly increased in KBALB incubated in DMEM4. Therefore, modification of the active cathepsin level is a preponderant factor in the Glc-dependent control of lysosomal proteolysis in KBALB cells.

DISCUSSION

The oncogene *ras* presents critical functions in carcinogenesis [23] and is implicated in many tumour types [24]. Fibroblasts transformed by the *ras* oncogene have therefore been extensively used as models to investigate tumorigenesis [49]. Protein turnover in cells transformed by the *ras* oncogene, like in other models of cancer cells, depends on cell density and is regulated by the concentration of serum or insulin [16,17,50]. The present results establish that, in *ras*-transformed cells, at high density and despite the presence of serum growth factors, Glc on its own is a nutrient essential for the control of protein turnover.

Several proteolytic pathways are implicated in intracellular protein breakdown: cytosolic proteasomes and calpains, and

lysosomal cathepsins. Among them, the ubiquitin–proteasome-dependent proteolytic pathway is believed to play a major role in the control of proteolysis in host tissues. In muscle wasting, which is a major feature of cancer cachexia, enhanced ubiquitin–proteasome-dependent proteolysis accounts for protein loss [3,4]. On the other hand, the lysosomal pathway has consistently been implicated in tumour and transformed cells. In fact, modifications of rates of protein degradation in transformed cells, whether due to cell density or serum concentration, were usually correlated with the lysosomal proteolytic capacity [51].

The present data support a preponderant role for the lysosomal pathway in the Glc-dependent control of intracellular proteolysis in KBALB cells. Glc did not alter the non-lysosomal proteasomal and Ca^{2+} -dependent proteolytic pathways, and neither lactacystin, a selective inhibitor of the proteasome [44], nor E-64d, which inhibits calpains [45], prevents Glc induction of proteolysis. By contrast, lysosomal proteolysis, which requires active cysteine (cathepsins B and L) and aspartic (cathepsin D) endopeptidases, was found to be determinant for the regulation by Glc of proteolysis. Strikingly, partial inhibition of lysosomal proteolysis (of cysteine endopeptidases by E-64d) was not sufficient, and Glc-dependent proteolysis was only hindered after maximal inhibition of both classes (cysteine and aspartic) of lysosomal cathepsins.

Schematically, lysosomal-dependent proteolysis involves the initial sequestration of protein substrates into the vacuolar system and their subsequent hydrolysis by lysosomal peptidases. Different pathways may be involved to deliver intracellular protein substrates to lysosomes. Cytosolic proteins enter the lysosome compartment by microautophagy (invagination of the lysosomal membrane), or by direct transfer through the lysosomal membrane when they contain a KFERQ motif [52]. Macroautophagy engulfs whole portions of cytoplasm together with various organelles. Crinophagy targets secreted proteins to lysosomes, while membrane proteins may reach lysosomes by endocytosis. In many cell types, the lysosomal endopeptidases apparently far exceed the needs for protein degradation, and the rate-limiting step is the sequestration of substrates into the vacuolar system. In transformed cells, modifications in rates of proteolysis were attributed to their macroautophagic capacity [21,22], but also to lysosomal endopeptidase activities [51].

Macroautophagy is the most appropriate mechanism for a rapid modulation of proteolysis by nutrients, and is indeed highly stimulated by starvation [53]. In the present case, however, rates of proteolysis were higher in medium containing more Glc, which does not correspond to nutrient deprivation. Although we do not know which pathway prevails in the targeting of substrates to lysosomes in KBALB cells, direct measurements of the vacuolar sequestration of a cytosolic enzyme (lactate dehydrogenase) indicated that sequestration was not altered by extracellular Glc. In contrast, Glc strongly increased the active forms of endosomal–lysosomal cathepsins. The selectivity of Glc effect on cathepsin expression in KBALB cells is particularly remarkable, as Glc does not alter other lysosomal non-proteolytic hydrolases [27] and other non-lysosomal endopeptidases (the present study). Altogether, these observations strongly support the notion that the Glc control of proteolysis in *ras*-transformed cells mostly depends on selective variations in the levels of endosomal–lysosomal active cathepsins. Glc strongly increases active forms of cathepsins L and B, without altering the levels of the mRNAs encoding these endopeptidases [27]. The post-transcriptional mechanisms implicated encompass increased translation and processing of procathepsin L [27], but also an enhanced stability of active cathepsins inside endosomes–lysosomes (C. Tournu, A. Obled, M.-P. Roux and D. M. Bchet,

unpublished work). The metabolic intermediate allowing Glc to control cathepsin expression remains to be specified. However, Glc transport and metabolism are required to increase cathepsin expression, as only metabolized sugars mimic the Glc-dependent increase in active cathepsins in KBALB cells [27].

The abundant literature on cathepsin expression in cancer cells is essentially based on their involvement in metastasis (reviewed in [49]), but rarely on their potential role in intracellular proteolysis [51]. The degradation of the extracellular matrix involves cascades of proteolytic enzymes, including collagenases, stromelysin, plasmin, plasminogen activator and cathepsins. In fact, increases in transcription, secretion of procathepsins, and altered localization of cellular cathepsins have all been reported to parallel malignant progression. Part of the active cathepsins are located in early endosomes and/or bound to the plasma membrane and are thus believed to participate in extracellular-matrix degradation [49]. Although Glc does not modify procathepsin L secretion in KBALB cells, it strongly increases active cathepsins in endosomal–lysosomal fractions [27], which may favour metastasis. The present study emphasizes that, in addition to their role in metastasis, endosomal–lysosomal cathepsins play another critical function in the control of intracellular proteolysis in transformed cells. Gradients of growth factors and nutrients, including Glc, exist within tumours, and we have herein established that Glc availability participates in this control of protein turnover.

What is the function of a Glc-dependent control of proteolysis in transformed cells? When cancer cells are closed to vessels, Glc enables high rates of protein turnover to occur, which possibly serve to tailor their phenotype to new environmental demands, and favour their metastatic intra- and extra-vascularisation. Alternatively, inside tumours, low Glc concentrations decrease active cathepsins and intracellular proteolysis. Low rates of lysosomal autophagic proteolysis have repeatedly been described in transformed and tumour cells, and assumed to provide them with selective advantages [21,22,51]. Emphasis was recently placed on this topic, as expression of *beclin-1*, an autophagy-promoting gene, was found to be commonly decreased, together with protein breakdown, in human breast carcinoma cells [22]. In fact, a reduced autophagic proteolysis appears as a fundamental mechanism which favours tumour-forming potential. The mechanisms involved have not been clarified, but transformed cells do not serve the needs of the organism and may suppress autophagic proteolysis to improve their ability to survive. A reduced autophagic proteolysis can result not only from a decreased sequestration, but also from a lower proteolytic capacity of lysosomes. Low Glc concentrations in tumour micro-regions may provide cancer cells with a regulated mechanism to similarly decrease lysosomal cathepsins and proteolysis and to favour tumorigenesis.

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