Published in final edited form as:

Brain Res. 2007 May 4; 1144: 231–238.

Extracellular glutamine is a critical modulator for regulatory volume increase in human glioma cells

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Abstract

Mammalian cells regulate their volume to prevent unintentional changes in intercellular signaling, cell metabolism, and DNA integrity. Intentional cell volume changes occur as cells undergo proliferation, apoptosis, or cell migration. To regulate cell volume, cells use channels and transport systems to flux osmolytes across the plasma membrane, followed by the obligatory movement of water. While essentially all cells are capable of regulatory volume decrease (RVD), regulatory volume increase (RVI) mechanisms have only been reported in some cell types. In this investigation, we used human glioma cells as a model system to determine conditions necessary for RVI. When exposed to hyperosmotic conditions through the addition of 30mOsm NaCl or sucrose, D54-MG and U251 glioma cell lines and glioma cells from acute patient biopsies shrunk transiently but were able to fully recover their original cell volume within 40–70minutes. This ability was highly temperature sensitive and absolutely required the presence of low millimolar concentrations of L-glutamine in the extracellular solution. Other known substrates of glutamine transporters such as methyl-amino isobutyric acid (MeAIB), alanine, and threonine were unable to support RVI. The ability of cells to undergo RVI also required the presence of Na^+ , K^+ , and Cl^- and was inhibited by the NKCC inhibitor, bumetanide, consistent with the involvement of a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC). Moreover, the expression of NKCC1 was demonstrated by Western blot. We concluded that regulatory volume increase in human glioma cells occurs through the uptake of Na^+ , K^+ , and Cl^- by NKCC1 and is modulated by the presence of glutamine.

Keywords

glutamine; regulatory volume increase; RVI; glioma; NKCC

1. INTRODUCTION

It is well established that volume regulatory mechanisms are necessary to help cells overcome the deleterious consequences that result from alterations in plasma osmolarity. Such changes in osmolarity occur, for example, in conjunction with diabetes mellitus, congestive heart failure, hyperaldosteronism, cirrhosis, enteric and renal disorders [11;25;33;36]. In the absence of cell volume regulation, the resulting volume changes may lead to alterations in metabolism, disrupt signaling pathways, cause DNA damage, and cause cells to be more susceptible to cell death [5;25;29]. In the brain, volume regulation is particularly important as perturbations in cell size alters the ionic composition of the extracellular space surrounding neurons and glia

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cells, thereby affecting normal brain function[33]. While volume regulatory mechanisms that occur under pathophysiological conditions have been well investigated, it is now apparent that these same mechanisms play equally important roles in “normal” physiological functions. For example, several studies suggest that cell volume regulation may be important in determining the rate of cell proliferation [2;8;22], in aiding cell migration [27;32] and regulating apoptotic programmed cell death [10;21].

Proliferation, migration, and apoptosis are especially important cellular traits of primary brain tumors (gliomas), central nervous system tumors derived from glial cells. Their poor prognosis is due to a high rate of proliferation and the marked ability to infiltrate into normal peritumoral brain. Glioma cell invasion is facilitated by cell volume decreases that occur through volume-regulatory chloride efflux [10;27;32]. Similar mechanisms have also been demonstrated in the local invasion of cervical cancer cells[31].

While mechanisms of volume decrease have been well studied in gliomas, very little is known about how cells increase their volume in physiological situations or in response to a challenge to volume homeostasis. Regulatory volume increase (RVI) can be induced by hyperosmotic challenge and is a prominent feature of epithelial cells, lymphocytes, and skeletal muscle [25;38]. In these cell types, cells shrink following a hyperosmotic challenge but are able to regulate their cell volume by activating the inward transport of osmolytes, including both organic osmolytes (betaine, taurine, glutamine, etc.) and inorganic ions (Na^+ , K^+ , Cl^- , etc.) followed by the obligatory movement of water [18;19;]. Most commonly, this osmolyte transport is thought to be accomplished through the action of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC), Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange, and chronic uptake through organic osmolyte transporters BGT1, TAUT, etc [18;19;25]. In contrast to regulatory volume decrease (RVD) mechanisms, which appear to be present in all cell types, not all cell types appear to be able to undergo RVI [19]. In glioma cells, limited studies have provided contrasting evidence for and against the presence of volume increase mechanisms. In the few studies demonstrating RVI in glioma cells, RVI was bumetanide-sensitive, suggesting that it was primarily due to osmolyte uptake through an NKCC [23;24;34]. However, other studies failed to demonstrate a significant volume increase after a hyperosmotic stress [3;17]. The reason for this discrepancy remains unclear. The majority of these prior studies were performed on C6 glioma cells, a cell line generated from a malignant glioma in rats. The mechanisms of volume increase, if they exist, in human glioma cells are unknown. In the following investigation, we demonstrate that human glioma cell lines as well as glioma cells isolated from patient biopsies have the ability to undergo regulatory volume increases. In addition, we conclude that glioma RVI occurs by Na^+ , K^+ , Cl^- uptake through bumetanide sensitive cation-chloride cotransporters but only in the presence of extracellular glutamine.

2. RESULTS

Glioma cells undergo a regulatory volume increase (RVI) in response to a hyperosmotic challenge

To determine whether or not glioma cells regulate their volume in the face of a hyperosmotic challenge, we obtained Coulter Counter measurements from D54-MG and U251 cell lines in the presence of an osmotic challenge. After baseline volume measurements (V_{baseline}) were recorded for 5min in the presence of normosmotic culture medium (DMEM/F12 supplemented with 7% BGS and 2mM gln) or normal NaCl bath solution, the cells were exposed to a 30mOsm increase in osmolarity by the addition of 15mM NaCl. Initially, the cells responded to the increase in osmolarity by shrinking to approximately 91% of their original volume, as expected for cells behaving like a perfect osmometer. While cells in NaCl bath solution remained shrunken in the sustained presence of the hyperosmotic challenge, cells suspended in culture medium began to undergo a rapid volume increase, recovering to their baseline volume within

30–60 minutes after the challenge (Fig. 1A and 1B). These data demonstrate that in order for glioma cells to be able to undergo a regulatory volume increase (i.e. to be RVI-competent) they require an additional factor that is present in the culture medium but is absent from normal bath solution. To rule out that this phenomenon is a property acquired uniquely by chronically cultured glioma cell lines, we repeated these experiments in cells acutely cultured from patient biopsies (labeled GBM50) and obtained identical results (Fig. 1C)

When exposed to challenges greater than 15mM NaCl, D54-MG cells in supplemented culture medium continued to initially shrink to a volume expected for perfect osmometers (Fig. 2B). Even though partial volume recovery occurred during each challenge, cells exposed to challenges greater than 30mM NaCl were unable to recover their baseline volume within 60 minutes (Fig. 2A).

Glutamine is both necessary and sufficient for RVI-competency in glioma cells

We sought to determine which of the culture medium components, not present in the normal bath solution, were necessary for the RVI competency of glioma cells. To this end, we examined RVI in D54-MG cells suspended in supplemented culture medium, DMEM/F12 alone, normal bath supplemented with 2mM glutamine (gln), normal bath supplemented with 7% BGS, and normal bath supplemented with Dubelco's vitamin solution (data not shown). As shown in Figure 3A, neither cells suspended in unsupplemented DMEM/F12 nor in normal bath supplemented with BGS were able to recover their initial volume after exposure to a hyperosmotic challenge. In contrast, cells suspended in normal bath supplemented with 2mM gln alone demonstrated the same degree of RVI as cells suspended in supplemented culture medium (Fig. 3A), suggesting that glutamine is both necessary and sufficient to render glioma cells RVI competent. To further determine the dose of glutamine required for glioma RVI, we examined RVI at several concentrations of glutamine ranging from 0.2 to 10mM. By plotting the %RVI (normalized to the %RVI in the presence of 10mM glutamine), we generated a dose dependent curve from which we extrapolated the EC₅₀ for glutamine to be approximately 0.75mM (Fig. 3B).

Glutamine-uptake through amino acid transporters is not responsible for RVI

Although it has been demonstrated that the regulatory volume decrease of glioma cells in the presence of a hyposmotic challenge is due to the efflux of chloride and most likely potassium [10], glutamine may be acting as the primary osmolyte in RVI. To determine if glutamine uptake through known amino acid transport systems was responsible for RVI, we repeated the above experiments in normal bath supplemented with other known substrates for glutamine transporters. However, none of the other substrates examined, including alanine, cysteine, histidine, MeAIB, or threonine, were sufficient to render the glioma cells RVI-competent (Fig. 3C). In addition, the D enantiomer of glutamine was also unable to substitute for the physiological L-glutamine in volume regulation. While the function of glutamine in RVI is not due to transport through known glutamine transporters, the necessity for the L enantiomer suggests that the structure of glutamine is very important.

Na⁺/K⁺/Cl⁻ cotransporters are responsible for osmolyte uptake during RVI

Although the unusual requirement for glutamine in RVI suggests a possible role for other osmolytes in addition to ions, previous studies in other cells have observed that the transport of Na⁺, K⁺, and Cl⁻ ions into the cell may also play a role in RVI. In rat gliomas cell lines, these studies have specifically suggested that RVI is primarily due to the uptake of these ions via the Na⁺/K⁺/Cl⁻ cotransporter, NKCC1. To ascertain if this transporter plays any role during RVI in human glioma cells, we took advantage of three properties of the NKCC: ion selectivity, temperature sensitivity, and inhibition by the loop diuretic, bumetanide. We, first, suspended the cells in bath solutions with Na⁺, K⁺, or Cl⁻ replaced by other ions (choline, Cs⁺, and

NO₃⁻, respectively) that are not easily transported by the NKCC [13]. As demonstrated in Figure 4A, each of these ion replacements resulted in the elimination of the ability of glioma cells to undergo RVI. Next, we challenged the cells, in the presence of glutamine, by a 30mOsm addition of NaCl while maintaining the cell suspensions at a temperature of 22°C or 37°C. In support of a role for a temperature-dependent osmolyte uptake system in RVI, the cells maintained at the lower temperature were unable to undergo a regulated volume increase (Fig. 4B). Finally, treating the cells with the relatively specific NKCC inhibitor bumetanide at 200μM or 20μM (data not shown) resulted in a significant inhibition of RVI (Fig. 4C). These data indicate that, in addition to glutamine, the presence of Na⁺, K⁺, and Cl⁻ are all necessary for RVI in glioma cells and that the NKCC is one of the major routes for the uptake of these ions.

The NKCC family is composed of two members, NKCC1 and NKCC2. To determine which of these transporters is likely responsible for the bumetanide-sensitive component of RVI, we investigated the protein expression of NKCCs in glioma cells. Western blots probed with commercially-available antibodies against each of the NKCC members revealed expression of NKCC1, but not NKCC2 in each of the glioma cell lines tested (Fig. 5).

3. DISCUSSION

Using dynamic cell size measurements, we demonstrate that human glioma cells have the ability to rapidly alter their cell size through regulatory volume increase after exposure to a hyperosmotic challenge. This occurs through osmolyte uptake, primarily via the Na⁺/K⁺/Cl⁻ cotransporter NKCC1. We further demonstrate that both cultured glioma cell lines and cells acutely cultured from patient biopsies were able to undergo RVI when exposed to a hyperosmotic challenge. This finding agrees with two previous studies in C6 glioma cells [23;24], but contradicts at least two other studies which concluded that glioma cells, and C6 rat glioma cells in particular, are unable to volume regulate in response to a hyperosmotic challenge [3;17]. One previous paper argued that the discrepancy between these studies may be due to temperature differences between experiments, as transport-mediated osmolyte uptake is highly temperature sensitive [24]. Although we were able to demonstrate that volume regulation in human glioma cells is temperature sensitive, we have also been able to show that other factors were necessary for volume regulation even when cell suspensions were maintained at 37°C. Surprisingly, we discovered that the presence of glutamine was necessary for glioma cells to undergo RVI. The effect of glutamine on the ability of glioma cells to undergo a regulated volume increase was shown to be dose-dependent, with an EC₅₀ of 0.75mM, which is within the range of the estimated glutamine concentration in the brain (0.37–0.83mM) [9;16]. To our knowledge, this result has not previously been reported. However, a further review of the literature indicated that the investigations in which glioma cells were found to be RVI-competent were performed in the presence of glutamine-containing bath solutions or media [23;24]. Similarly, studies that demonstrated RVI-incompetency were performed in the absence of glutamine. Hence, while these studies may have hinted at the involvement of glutamine in glioma cell RVI, this had not yet been experimentally evaluated. By contrast, glutamine has been experimentally shown to limit the detrimental effects that exposure to a hyperosmotic challenge may have to other cells and tissues, including an increased susceptibility to apoptosis [14;26]. For example, in cardiac transplants where the hearts are often stored in hypertonic saline, cell death can be attenuated by supplementing the hypertonic solution with glutamine [26].

In human glioma cells, post-hyperosmotic regulated cell swelling may occur through glutamine accumulation through transporters and a subsequent increase in intracellular osmolyte concentration. Indeed, in fibroblasts, the uptake of glutamine and other amino acids through the amino acid transporter, system A, has been shown to increase during a hyperosmotic

challenge [4]. In the brain, four amino acid transport systems are available for the transport of glutamine into and out of cells including systems A, ASC, L, and N. These transporters can be distinguished based on their Na^+ -dependence (systems A, ASC, and N are Na^+ -dependent), pH-inhibition (systems A and N), and diverging substrate affinities[1]. In C6 glioma cells, glutamine uptake has been shown to be primarily due to system ASC, and partially to system N, transporters [1;6;7]. If glutamine uptake through one of these transporters is responsible for RVI in glioma cells, then we hypothesized that other substrates for these transporters should be able to serve as osmolytes in the absence of glutamine and promote RVI. However, we discovered that neither substrates for system A (alanine, MeAIB), system ASC (alanine, cysteine, threonine), system L (histidine) nor system N (threonine, histidine) were sufficient for volume recovery after a hyperosmotic challenge.

In astrocytes, glutamine has been shown to be involved in ammonia-induced swelling, as may occur during hepatic failure, Reye's syndrome, or urea cycle disorders. The mechanism for ammonia-induced astrocytic swelling has been shown to involve an increase in cortical glutamine due to the action of glutamine synthetase and the subsequent hydrolysis of glutamine by intracellular phosphate-activated glutaminase (PAG) [15;35;37]. Glutamine hydrolysis and cell swelling can be inhibited by treatment with the relatively specific drug, DON [15]. Unlike these astrocytes, however, in the absence of a hyperosmotic challenge, an increase in glutamine, alone, was not sufficient to result in cell swelling of cultured glioma cells. Furthermore, pretreatment of glioma cells with DON did not inhibit RVI in cells challenged hyperosmotically (data not shown), suggesting that phosphate-activated glutaminase is not involved in glutamine-mediated regulatory volume increase.

While these data indicate that glutamine involvement in glioma RVI is not due to uptake through known glutamine transporters or due to subsequent glutamine hydrolysis, many other possibilities exist that may explain the necessity of glutamine. First, it is possible that a previously undescribed glutamine transporter with a narrower substrate specificity may be responsible for osmolyte uptake. However, the complete lack of RVI, even through known transport pathways such as the NKCCs, in the absence of glutamine suggests that this amino acid may be serving as an allosteric modulator to other osmolyte flux pathways, or simply that the lack of this necessary metabolite may disrupt necessary signaling pathways involved in RVI. Further investigations are necessary to determine what role glutamine plays in volume regulation.

While our data support a primary role for glutamine in RVI, studies in other cell types have concluded that osmolyte uptake may also occur through ion transporters including the $\text{Cl}^-/\text{HCO}_3^-$ exchanger and the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter (NKCC). Because previous studies in rat glioma cell lines focused mainly on the NKCC, we chose to narrow our investigation to the role of NKCCs in human glioma cell RVI. To this end, all of our experiments were performed in the presence of HEPES buffered bath solution, precluding any role of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Similar studies performed on C6 glioma cells in the presence of glutamine concluded that osmolyte uptake during RVI occurred primarily through an NKCC [23;24;33], analogous to RVI in other cell types [12;20;28;30]. In support of this conclusion, human glioma cells were unable to undergo RVI in the absence of external Na^+ , K^+ , or Cl^- ions required for NKCCs to be functional [13]. Furthermore, our data indicate that, as measured at 40' after challenge, 57% of RVI ($p=0.03$) could be inhibited in the presence of 20 μM bumetanide, a concentration that is specific for inhibition of NKCC, and 85% of RVI could be inhibited in the presence of 200 μM bumetanide ($p=0.007$). However, even in the presence of high concentrations of bumetanide, inhibition of RVI was not complete, allowing for the possibility that other osmolyte transporters may contribute to the process, including Na^+ -dependent amino acid transporters. Our data further indicate that only NKCC1, and not NKCC2, is expressed in human glioma cells. These results are consistent with previous

investigations of the protein expression profile of these two proteins which have concluded that NKCC1 is expressed in many cells and tissues while NKCC2 is most often only found in kidney cells [28]. Therefore, we conclude that NKCC1 contributes to osmolyte uptake during RVI in glioma cells.

Overall, in addition to demonstrating that glioma cells are able to undergo a regulatory volume increase in the presence of a modest and physiologically relevant hypertonic challenge, the most provoking new observation in this study is that RVI in human glioma cells is glutamine-dependent. In addition to their importance in helping cells to recover from otherwise detrimental osmotic stressors, volume regulatory mechanisms (both volume increase and decrease) have been implicated in other physiological processes including migration, apoptosis, proliferation, and volume homeostasis in the presence of metabolic changes. The requirement for glutamine in RVI suggests that it may play a yet unidentified role in the above processes as well, a hypothesis which deserves further investigation.

4. EXPERIMENTAL PROCEDURE

Cell Culture

All experiments were performed using the glioma cell lines D54MG (glioblastoma multiforme, WHO grade IV; Dr. D. Bigner, Duke University) and U251 (GBM, WHO grade IV, Dr. Y. Gillespie, University of Alabama at Birmingham) and cells acutely cultured from patient biopsies (GBM50; glioblastoma multiforme, WHO grade IV; Dr. Y. Gillespie). Cell cultures were maintained in DMEM/F12 (Life Technologies, Grand Island, NY) supplemented with 2mM L-glutamine (CellGro, Herndon, VA) and 7% bovine growth serum (HyClone, Logan, UT). The cells were kept at 37°C in a 90% O₂-10% CO₂ humidified atmosphere.

Cell Volume Measurements

Cell volumes were measured as previously described [10] by electronic sizing with a Coulter counter Multisizer 3 (Beckman-Coulter, Miami, FL). The aperture size used for these experiments was 100- μ m.

To prepare the cells for acute volume measurements during regulatory volume increase experiments, cells were incubated for 3 minutes with 0.05% trypsin and 0.53mM EDTA (Invitrogen Corporation, Carlsbad, CA). Trypsin was inactivated by the addition of an equal volume of serum-containing medium. After a brief centrifugation, the cells were resuspended in pre-warmed bath solution or culture medium and passed through a 40- μ m nylon cell strainer (Fisher). Cells were incubated in bath solution for 10 minutes before the beginning of the first baseline measurement. Cell volume measurements were obtained every 2 minutes and each measurement was an average of 10,000–20,000 cells. Five or six baseline readings were collected before the osmotic challenge was applied. Cell suspensions were maintained at 37°C for the duration of all experiments unless otherwise specified.

Solutions

The control NaCl bath solutions (“normal bath”) contained the following (in mM): 130 NaCl, 5.0 KCl, 10.5 glucose, 32.5 HEPES, and 1 CaCl₂. When indicated, the NaCl and KCl salts were substituted with NaNO₃ and KNO₃ (“Cl⁻-free bath”), NaCl was substituted with NMDG-Cl (“Na⁺-free bath”), and KCl was substituted with CsCl (“K⁺-free bath”). The pH of each solution was adjusted to 7.4 with NaOH and the osmolality of each solution was confirmed with a vapor pressure osmometer (Wescor 5500; Wescor, Logan, UT). The osmolalities of our solutions were 310 $\pm$ 10mOsM.

For experiments in which amino acid supplementation was required, the amino acid was added to the bath solution at a concentration of 5mM prior to adjusting the pH.

Drugs were added directly to bath solutions and culture media from stock solutions. Bumetanide was dissolved at 500X final concentration in DMSO. DMSO at its final concentration (0.2%), did not perturb cell volumes or affect volume regulation (data not shown).

Data analysis for volume regulation experiments

Coulter counter data were collected with Multisizer 3 software and size listings were exported to Excel. Time points were rounded to whole minutes and mean cell volumes were normalized to the average baseline value for that experiment. All data were plotted in Origin 7.0 (MicroCal, Northampton, MA) as means $\pm$ SE with the number of experiments performed (*n*). Significance (*p*) was determined by Student's *t*-test of relative volumes at 20, 40 and 60 minutes after challenge.

Western Blots

Cells in 100-mm culture dishes were rinsed with ice cold PBS, incubated in RIPA buffer supplemented with 1:500 protease inhibitor cocktail (Sigma) for 30 minutes, and mechanically lifted from the dish. The sample was then gently mixed for 30 minutes, and centrifuged at 4°C. The proteins of the supernatant were separated by electrophoresis using a 7.5% SDS-polyacrylamide gel (Bio-Rad). Proteins were transferred onto PVDF membranes (Millipore), probed with antibodies obtained from Alpha Diagnostics (San Antonio, TX) and visualized using HRP-conjugated anti-rabbit antibodies (BIO-RAD) and ECL system (Amersham).

Acknowledgements

This work was supported by grants RO1-NS-36692 & RO1-NS-31234 from the National Institutes of Health.

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Figure 1. Glioma cells exposed to a hyperosmotic challenge undergo a regulatory volume increase in the presence of supplemented cell culture medium

Normalized mean cell volumes recorded during a hyperosmotic challenge induced by the addition of 15mM NaCl. D54-MG cells (A), U251 cells (B), and cells cultured acutely from a patient biopsy, labeled GBM50 (C), were suspended in normal NaCl bath solution or cell culture medium supplemented with 2mM glutamine and 7% bovine growth serum. ($p < 0.05$ at 20, 40, or 60 minutes is indicated by *.)

Figure 2. The ability of D54-MG cells to undergo RVI is independent of the extent of the hyperosmotic challenge

A: Normalized mean cell volumes recorded during a hyperosmotic challenge induced by the addition of various concentrations of NaCl. **B:** Normalized mean cell volumes recorded at the initiation of the hyperosmotic challenge (V_0 , both actual observed and those expected for a perfect osmometer) compared to the volumes attained after 60 minutes of volume regulation (V_{60}).

Figure 3. Extracellular glutamine is necessary for RVI in D54-MG cells

A: Normalized mean cell volumes of glioma cells were recorded during a hyperosmotic challenge induced by an addition of 15mM NaCl. Cells were suspended in unsupplemented cell culture medium, cell culture medium supplemented with glutamine and bovine growth serum, normal NaCl bath solution supplemented with glutamine alone, or normal NaCl bath solution supplemented with bovine growth serum alone. **B:** A dose-response curve was generated by plotting the maximum RVI response observed versus the concentration of extracellular glutamine at which the observed response occurred. **C:** *Known glutamine transport systems are not involved in RVI of D54-MG cells* Normalized mean cell volumes at the initiation of the hyperosmotic challenge (0') compared to those recorded after 60' of volume

regulation in the presence or absence of 5mM amino acids or amino acid derivatives. When compared to bath alone, $p < 0.05$ is indicated by *.

Figure 4. Ion uptake through a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter (NKCC) is necessary for RVI in D54-MG cells

Normalized mean cell volumes recorded during a hyperosmotic challenge induced by the addition of 15mM NaCl. **A:** A comparison of the response of cells maintained at 37°C versus room temperature (R.T.). **B:** A comparison of the response of cells suspended in normal NaCl bath versus those suspended in bath solutions lacking extracellular Na^+ , K^+ , or Cl^- . **C:** Volumes recorded at the initiation of the hyperosmotic challenge (0') compared to those recorded after 40' of volume regulation in the presence or absence of glutamine and the NKCC inhibitor, bumetanide. ($p < 0.05$ indicated by *.)

Figure 5. NKCC1 but not NKCC2 is expressed in human glioma cell lines and cells from a patient biopsy

Whole cell lysates from D54-MG and U251 cells as well as the patient sample GBM50 were separated by SDS-PAGE and probed with commercial antibodies generated against NKCC1 and NKCC2.