Up-Regulation of ALG-2 in Hepatomas and Lung Cancer Tissue

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ALG-2 was isolated in a screen for proteins involved in programmed cell death and is the first Ca^{2+} -binding protein found to be directly involved in apoptosis. We have generated polyclonal antibodies that are suitable for detecting ALG-2 using different immunological methods. Three commercial antibodies against ALG-2 did neither detect mouse recombinant ALG-2 nor endogenous ALG-2 in Jurkat cell lysates, whereas our own affinity-purified antibody recognized recombinant as well as endogenous ALG-2. The specificity of the antibody was shown by preabsorption experiments and on ALG-2-deficient cells using Western blot analysis and immunohistochemistry. Western blot analysis of 15 different adult mouse tissues demonstrated that ALG-2 is ubiquitously expressed. We found that ALG-2 was more than threefold overexpressed in rat liver hepatoma compared to normal rat liver using Western blot analysis, a result confirmed by immunohistochemical analysis. Staining of four different lung cancer tissue microarrays including specimens of 263 patients showed that ALG-2 is mainly localized to epithelial cells and significantly up-regulated in small-cell lung cancers and in non-small-cell lung cancers. Our results lead to the conclusion that ALG-2 beside its known proapoptotic functions may be a player in survival pathways. (*Am J Pathol* 2003, 163:81–89)

Cancer of the lung and bronchus is the second most frequent cancer among both genders in the United States comprising >13% of all cancer incidents and responsible for >29% of all cancer deaths, thus by far the major cause of cancer mortality.¹ The high lethality of lung cancer is partly because of the lack of efficient early stage detection methods and despite an increase in population screening, 85% of those diagnosed with lung cancer are diagnosed in late stage. It is therefore of major interest that prognostic tools such as molecular markers are found and used for clinical purposes.

In the search for molecular markers it is relevant to focus on proteins involved in cell proliferation and apoptosis, as it is often observed that dysregulation of these important cellular processes leads to neoplastic growth. The Ca^{2+} -binding protein ALG-2, the product of apoptosis linked gene-2, was identified in a screen for genes coding for proteins involved in apoptosis.² Mouse T-cell hybridoma 3DO transfected with anti-sense *alg-2*, was desensitized toward stimulation with Fas antibodies and glucocorticoids, which are known to activate caspase-dependent apoptotic pathways.² Yet, no decrease in caspase activity was seen in the cell death resilient *alg-2* anti-sense clones and it was, therefore, suggested that ALG-2 functions downstream or parallel to the caspases in the apoptotic pathway.³ Two groups independently found by yeast two-hybrid screenings that ALG-2 interacts with the SH3-binding domain containing protein AIP1 (ALG-2-interacting protein-1)⁴ or Alix (ALG-2 interacting protein x).⁵ AIP1/Alix shares homology with a putative signal transduction protein, BRO1, from *Saccharomyces cerevisiae*⁶ and is an orthologue of an *Xenopus laevis* protein, XP95, involved in cell-cycle regulation.⁷ It was shown by overexpression experiments that ALG-2 interacts with AIP1 in a Ca^{2+} -dependent way,⁵ a result confirmed *in vitro* by another group.⁴ This points to the function of ALG-2 and AIP1 in a novel Ca^{2+} -dependent signaling pathway. Overexpression of a 402-amino acid-long C-terminal fragment, TH28, of AIP1 in HeLa and COS cells delayed cell death induced by either etoposide, staurosporine, or serum starvation.⁴ Co-transfection of ALG-2 and TH28 not only abrogated the protective effect of TH28, but augmented effects of etoposide or staurosporine.⁴ However, recent investigations on ALG-2-deficient mice did not confirm the previously reported proapoptotic properties of ALG-2 which might be explained by redundancy.⁸

ALG-2 has been shown to interact with a two amino acid shorter splice variant, ALG-2.1, which does not bind AIP1 and shows lower affinity for Ca^{2+} .⁹ Evidence for

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heterodimerization of ALG-2 with peflin,¹⁰ annexin VII¹¹ and annexin XII,¹² all containing Ca^{2+} binding motifs, has been given recently. To examine the expression of ALG-2 by immunological methods we have made two polyclonal antibodies against ALG-2 and used them for immunohistochemical and Western blot analysis of protein extracts from rat normal liver and hepatomas. Further, we have used one antibody for immunohistochemical screening of small-cell lung cancers (SCLC) and non-small-cell lung cancers: squamous cell carcinomas (SCC), lung adenocarcinomas (LAdC), and large-cell lung cancers (LCLC), arranged in tissue microarrays. All surgical specimens of cancerous tissues showed a marked increase in ALG-2 expression compared to the adjacent normal tissue. These findings indicate that ALG-2 may play other roles than the proapoptotic one described so far.

Materials and Methods

Antibody Production and Affinity Purification

ALG-2.1 and ALG-2 cDNAs were cloned from mouse liver RNA by reverse transcriptase-polymerase chain reaction and inserted into the pGEMEXII vector as described previously.⁹ Recombinant ALG-2 was expressed in *Escherichia coli* BL21(DE3) by induction of expression with 0.5 mmol/L of isopropyl-beta-D-thiogalactopyranoside (IPTG) and purified by Ca^{2+} precipitation followed by anion exchange chromatography in a 0- to 500-mmol/L NaCl gradient.⁹ Antiserum against full-length protein was made by subcutaneous injection of purified recombinant ALG-2.1 and ALG-2 in a 1:1 mixture into rabbits (Dako-Cytomation, Glostrup, Denmark). Anti-peptide serum against ALG-2 was made by injection of rabbits with peptides corresponding to amino acids 117 to 124 of ALG-2.1 or amino acids 117 to 133 of ALG-2 linked to carrier protein diphtheria toxoid or keyhole limpet hemocyanin, respectively (Chiron, Emeryville, CA). Preceding affinity purification of antisera 0.75 mg of ALG-2.1 or 1 mg of ALG-2 were coupled to 0.4 g NHS-activated Sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden). After conjugation in a coupling buffer (0.1 mol/L NaHCO_3 and 0.5 mol/L NaCl, pH 8.3) the ALG-2 Sepharose was washed on a glass filter with coupling buffer followed by a 0.1 mol/L Tris-HCl, pH 8.0, wash. ALG-2 Sepharose was transferred to a 14-ml tube and nonreacted groups were further blocked by 2 hours of incubation in 0.1 mol/L of Tris-HCl, pH 8.0, followed by washing in 50 ml of 0.1 mol/L NaOAc-HAc, pH 4, five times and in 50 ml of 0.5 mol/L NaCl and 0.1 mol/L of Tris-HCl, pH 8.0, five times. The ALG-2-coupled Sepharose was mixed with rabbit antiserum and incubated for 2 hours in coupling buffer and then loaded onto a column (10 cm, 1 cm in diameter). After washing with 100 ml of coupling buffer the purified antibody was eluted in 10 ml of 0.1 mol/L glycine-HCl, pH 2.5, and the pH of the collected fractions was adjusted to pH 7.8 with 1 mol/L of Tris-HCl, pH 8.0. The antibody fraction derived from the serum of peptide immunized rabbits was named P1 (0.14 mg/ml) and the

fraction derived from rabbits immunized with recombinant ALG-2 was named B1 (0.1 mg/ml).

Cell Cultures

The human T-cell lymphoma cell line, Jurkat E6-1 (a gift from Bela Papp, INSERM, Paris, France), was cultured at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Jurkat cells were grown to a cell density not exceeding 1.5×10^6 cells per ml. To prepare total cell lysates for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses, cells were washed once in PBS and then lysed in a RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.1% SDS, 0.5% Na-deoxycholate, 150 mmol/L NaCl, 0.02% NaN_3) and 1:1000 protease inhibitor cocktail (Sigma, St. Louis, MO). The protein extracts were centrifuged at $15,000 \times g$ for 10 minutes at 4°C and the supernatant used for SDS-PAGE. DT40 chicken B-cell lymphoma cells, kindly provided by Dr. Tomohiro Kurosaki (Kansai Medical University, Osaka, Japan), were propagated at 40°C in a 5% CO_2 atmosphere in RPMI 1640 with L-glutamine, 10% fetal calf serum, 1% chicken serum, 2 mmol/L L-glutamine, penicillin, and streptomycin.

Tissues and Tissue Microarrays

Cancerous and corresponding normal tissue from 263 lung cancer patients were obtained from the Department of Pathology, Rigshospitalet, Copenhagen, and processed for tissue microarray production. In brief, two representative 0.6-mm cores were punched from diagnostic areas of both tumor and normal tissue from the same patient, and inserted in a grid pattern in a single-recipient paraffin block using a tissue arrayer (Beecher Instruments, Silver Spring, MD) as previously described.¹³ Commercial tissue arrays, BA2, containing spots from 19 cancerous and 20 normal tissues were obtained from Bio-Cat, Heidelberg, Germany. DT40 ALG-2-deficient cells (P Winding and MW Berchtold, unpublished) and wild-type DT40 control cells were pelleted at $800 \times g$ for 5 minutes and coagulated with serum and thrombin. The cell coagulates were fixed in formalin for 4 hours and paraffinized overnight in the same block carrying both DT40 ALG-2-deficient cells and wild-type DT40 control cells. Rat liver and rat Morris hepatoma were paraffin-embedded in the same block then cut in 5- μm sections and picked up on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). For Western blot analysis, mouse tissues (Pel Freeze Biologicals, Rogers, AR) were pulverized in liquid nitrogen and boiling buffer containing 1% SDS, 1 mmol/L Na_3VO_4 , and 10 mmol/L Tris-HCl, pH 7.4 was added. Further homogenization was done by two times sonication for 15 seconds. Archival rat liver tissue and rat Morris hepatoma kindly provided by Clive A Slaughter (Howard Hughes Medical Institute, Dallas, TX) were pulverized in liquid nitrogen and homogenized with a Dounce homogenizer and triturated with a 23-gauge syringe in a protein extraction buffer containing

Figure 1. Specificity of ALG-2 antibodies. Immunoblot of Jurkat cell lysates from a 12% SDS polyacrylamide gel. The PVDF membrane was cut in 3-mm strips. Strips 1 to 4 and 5 to 8 were incubated with the H-14 and A-17 antibodies, respectively; diluted 1:100 in strips 1, 2, 5, and 6 and 1:1000 in strips 3, 4, 7, and 8. Strips 9 and 10 were incubated with the clone 22 antibody 1:5000. Strips 11 and 12 were incubated with our peptide antibody P1 diluted 1:500, and strips 13 and 14 with our antibody generated against recombinant ALG-2, diluted 1:1000. Antibodies in all even numbered strips were preabsorbed with an excess of recombinant ALG-2.

10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 5 mmol/L EDTA, 5 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton X-100, 20 mmol/L HEPES-KOH, pH 7.5, and 1:500 protease inhibitor cocktail. Both mouse and rat lysates were centrifuged at $10,000 \times g$ for 10 minutes and the protein concentrations were measured using a modified Lowry colorimetric assay (BioRad, Richmond, CA). After calibration of protein content the extracts were boiled for 5 minutes after addition an equal volume of sample buffer containing 100 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 200 mmol/L dithiothreitol.

Western Blot Analysis

Jurkat cell protein lysates were mixed with an equal volume of 4% SDS, 100 mmol/L Tris-HCl, pH 8.8, 30% glycerol, and 0.2% bromophenol blue, loaded in a single preparative lane and separated by 12% SDS-PAGE. The proteins were transferred to polyvinyl difluoride membranes (PVDF), Hybond-P (Amersham Biosciences, Uppsala, Sweden). The membranes were blocked with 5% skim milk in Tris-buffered saline (20 mmol/L Tris at pH 7.4, 140 mmol/L NaCl) and 1% Tween 20 for 1 hour, sliced in 3-mm strips, and incubated 1 hour with either of the following commercial anti-ALG-2 antibodies: H-14, A-17 (Santa Cruz Biotechnology, Santa Cruz, CA), clone 22 (BD Transduction Laboratories, Lexington, KY), and our affinity-purified antibodies B1 and P1. Dilutions are given in legend to Figure 1. The antibodies H-14 and A-17 are goat polyclonal antibodies directed against amino acids 130 to 142 or 51 to 67, respectively, of ALG-2 (sequences that are identical in human and mouse ALG-2). The clone 22 mouse monoclonal antibody has been made against a peptide comprising amino acids 162 to 181 of ALG-2

(with identical sequences in human and mouse ALG-2). Anti-ALG-2 antibodies from both Santa Cruz Biotechnology and BD Transduction Laboratories have been recommended for use in Western blot as well as for immunohistochemical analysis. Primary antibody incubation was followed by three washing steps and incubation with either anti-goat, anti-mouse, or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark). Horseradish peroxidase activity was detected using the ECL chemiluminescent reagent (Amersham Biosciences). Mouse tissues as well as rat liver (normal and Morris hepatoma) protein extracts were resolved by 12% SDS-PAGE and proceeded to Western blotting as above. For quantification of the endogenous ALG-2 in rat liver protein extracts, 0.25, 0.5, 1, and 2 ng of recombinant ALG-2 were loaded onto the same gels as standards. The quantification was performed using the NIH-image program.

Immunohistochemistry

Five- μm tissue microarray, DT40 coagulate sections, and paraffin-embedded liver tissue sections were stained, using the same procedure for all immunohistochemical experiments. Sections were deparaffinized in xylene and rehydrated in a series of ethanol dilutions. Antigen retrieval was obtained by microwave heating two times for 5 minutes at 500 W in 140 mmol/L of Tris-HCl, pH 9. Endogenous peroxidase activity was blocked for 10 minutes by incubation in 3% hydrogen peroxide. The sections were blocked for 30 minutes in Tris-buffered saline containing 2% bovine serum albumin (Sigma, St. Louis, MO) and incubated 1 hour at room temperature with 4 $\mu g/ml$ of B1 anti-ALG-2 antibody in the same buffer. For control experiments, tissue sections were either incubated without primary antibody or with primary antibody, which was preabsorbed for 90 minutes with an excess of recombinant ALG-2.1/ALG-2. The EnVision+ anti-rabbit IgG from DakoCytomation was used as a secondary antibody. Incubation with both primary and secondary antibody was followed by three 5-minute washes in Tris-buffered saline/0.1% Tween 20. The antibody-peroxidase complex was visualized using an enhanced diaminobenzidine (DAB) chromogen method, DAB+, according to the manufacturer's protocol (DakoCytomation). The sections were subsequently counterstained with Mayer's hematoxylin and dehydrated in ethanol and xylene before mounting with Eukitt mounting medium (Bie & Berntsen, Rødovre, Denmark).

Evaluation of Tissue Microarray Immunostaining

The intensity of the ALG-2 staining in the tissue microarrays was independently evaluated by three observers without clinicopathological experience to ensure nonbiased outcome. The intensities of the DAB+ staining were assigned arbitrary values from 0 to 4, where 0 corresponds to no staining and 4 to dark brown. The Wilcoxon signed rank test was applied to evaluate the significance of the intensity change between paired observations. An

observation pair comprises doublets of cancerous and corresponding normal lung tissue from the same patient. Furthermore, the scoring for all spots was reviewed for consistency, and intensity values deviating with more than one from two consistent scores were discarded as inconclusive.

Results

Specificity of Anti-ALG-2 Antibodies and Expression Pattern of ALG-2 in Different Mouse Tissues

Three different commercial anti-ALG-2 antibodies and two of our own affinity-purified antibodies against ALG-2 were tested in Western blot analysis using Jurkat cell lysates. Two of the commercial antibodies did not recognize any protein at the expected size of ALG-2 of 21.9 kD,² whereas one recognized a protein in Jurkat cells running ~2 kD higher than the endogenous and recombinant ALG-2 recognized by our own antibodies (Figures 1 and 4A). When using the commercial antibodies, no decrease in band intensity was seen after preabsorbing the antibodies with an excess of recombinant ALG-2, whereas the single band, recognized by the B1 antibody completely disappeared when incubating with an excess of recombinant ALG-2 (Figure 1, lane 14). The peptide antibody, P1, recognized a band running at the same size as the recombinant protein, and the signal slightly decreased after preabsorbing P1 with recombinant ALG-2 (Figure 1). Mouse recombinant ALG-2 was not recognized by the three commercial antibodies in a Western blot analysis (not shown). We have confirmed that the sequence of recombinant ALG-2 is identical to the published sequence of ALG-2 by tryptic digestion followed by mass spectrometry analysis (data not shown). ALG-2 expression levels were analyzed in 15 different archival mouse tissues by Western blotting (Figure 2). These data indicate that ALG-2 is ubiquitously expressed in different mouse organs (Figure 2). An additional band running below 22 kD was stained with anti-ALG-2 antibody in the submaxillary gland (Figure 2). This is most likely the result of proteolytic degradation during sample preparation.

Anti-ALG-2 Antibody in Immunohistochemistry

To test whether the anti-ALG-2 antibody, B1, is applicable for immunohistochemical analysis of formalin-fixed, paraffin-embedded tissue we made slides holding both DT40 wild-type and ALG-2-deficient DT40 cells. Before mounting the DT40 cells they were formalin-fixed and paraffin-embedded to mimic the handling of human archival material (Figure 3A, left). Wild-type DT40 cells showed strong immunoreactivity, whereas the ALG-2-deficient cells showed no staining (Figure 3A, right) confirming that the affinity-purified ALG-2 antibody, B1, is suitable for immunohistochemical experiments of paraffin-embedded material. Chicken ALG-2 is 81.7% identi-

Figure 2. Expression of ALG-2 in mouse tissues. Total protein from mouse tissues was extracted, calibrated (40 µg/lane), and run on a 20 × 18-cm 12% SDS gel before transfer to a PVDF membrane and immunolabeling with anti-ALG-2 antibody, B1.

cal to mouse ALG-2 and can also be detected by Western blot analysis using the B1 antibody (P Winding and MW Berchtold, unpublished). Furthermore, preabsorption of the antibody with recombinant ALG-2, in a molar ratio of 1:35 completely blocked the signal (Figure 3B), indicating that the signal is specific for ALG-2.

Expression of ALG-2 in Normal Livers and Hepatomas

To examine whether there may be differences in the expression of ALG-2 between normal and cancerous cells we used rat normal liver and rat Morris hepatoma as a model for normal and cancerous tissue. Protein extractions were made from normal liver and hepatoma specimens also processed for immunohistochemistry. To quantitate the expression of ALG-2 in cancerous tissue compared to normal tissue, we loaded tissue protein extracts and various amounts of recombinant ALG-2 on the same gel. By calculating band intensities using the NIH image program we found that ALG-2 levels are increased from ~30 ng of ALG-2/mg total protein in normal liver to ~100 ng/mg in hepatoma (Figure 4A). The recombinant ALG-2 ran in two bands. The lower band is most likely a degradation product of ALG-2 because the ratio of the higher band, which runs at the expected size *versus* the lower band, decreases during prolonged storage (Figure 4A). Both bands were used when ALG-2 levels in normal liver and hepatoma were quantified. Overexpression of ALG-2 in Morris hepatoma was further confirmed by immunohistochemical analysis of the tissues used for semiquantitation of ALG-2 expression (Figure 4B). In a commercial tissue array we found that ALG-2 is also up-regulated in human hepatocellular carcinomas compared to corresponding normal liver tissue samples (Figure 4C). The immunohistochemical staining of the tissue array showed that ALG-2 is also highly expressed in human sarcomas (not shown).

Figure 3. Specificity of ALG-2 antibody B1 in immunohistochemistry. **A:** Wild-type DT40 control cells (**left**) and DT40 ALG-2 knockout cells (**right**) were pelleted and coagulated with serum and thrombin. The cells were fixed in formalin for 4 hours, paraffinized, cut, and mounted on the same slides and ALG-2 was immunodetected using anti-ALG-2 B1 at 4 $\mu\text{g}/\text{ml}$. **B:** ALG-2 signal in human ovaries (**left**) and signal blocked by preabsorbing anti-ALG-2 antibody with an excess of recombinant ALG-2 (**right**). Scale bars: 50 μm (**A**); 200 μm (**B**).

Figure 4. Semiquantitative measurements of ALG-2 expression in rat liver and hepatoma. **A:** Lanes 1 to 4 represent proteins from different liver tissue extractions; lanes 1 and 3, normal liver, and lanes 2 and 4, hepatomas. Each lane was loaded with 14 μg of total protein. Vertical text indicates amounts in ng of recombinant ALG-2 used as a standard for quantifying endogenous ALG-2 in liver tissues. **B:** Immunohistochemistry of ALG-2, done on normal rat liver (**left**) and hepatoma (**right**) corresponding to lanes 3 and 4 in **A**. **C:** ALG-2 staining of normal human liver (**left**) and adjacent hepatocellular carcinoma (**right**) from commercial tissue array. Scale bar, 50 μm .

Table 1. Evaluation of ALG-2 Immunohistochemistry on Lung Tissue Microarrays

Total number of cases	Diagnosis	Paired observations			Wilcoxon signed rank test <i>P</i> value <	Nonpaired observations	
		Staining intensity		Intensity increase in %		Intensity increase in %	
		Normal	Tumor			Tumor	Metastasis
99	SCCL	2.3	3.3	42.7	0.00004	45.8	20.2
65	LCLC	2.3	2.8	21.6	0.00008	19.0	5.1
50	LAdC	2.5	3.4	34.4	0.00005	37.7	34.5
49	SCC	2.4	3.1	29.6	0.00006	31.1	10.2

Up-Regulation of ALG-2 in Lung Cancer Tissue

Based on the initial finding that ALG-2 is highly expressed in different cancers we investigated ALG-2 expression in lung carcinomas and adjacent normal tissue from 263 patients. By three independent observers it was found that the intensity of ALG-2 staining (Table 1) was higher in what was histologically determined as cancerous tissue than in the normal tissue. The intensity increased by 42.7% ($P < 0.00004$) in SCLC, whereas a 21.6% ($P < 0.00008$) increase in ALG-2 intensity was found in large-cell lung cancer (Table 1). The metastatic tissue was not analyzed using the Wilcoxon signed rank test because too few paired observations were available. The mean value of nonpaired observations did not show any increase in ALG-2 staining intensity in metastatic tissue derived from patients diagnosed with large-cell lung cancer, whereas a 34.5% increase in ALG-2 intensity was observed in metastatic tissue from lung adenocarcinomas patients (Table 1). ALG-2 was predominantly located to alveolar type II cells and macrophages, respiratory bronchial epithelia (Figure 5, A and B), or the bronchial glandular epithelia of normal tissue (Figure 5C). None or little ALG-2 staining was seen in the stromal areas of the cancerous tissue (Figure 5, D to F). In several cancer cells a solely cytoplasmic staining was seen (Figure 5D) whereas others exhibited predominant nuclear staining (Figure 5, E and F), however none showed exclusively nuclear staining. In normal cells both cytoplasmic staining and nuclear staining was observed (Figure 5, A to C).

Discussion

The proposed involvement of ALG-2 in apoptosis makes it a target for investigation of human malignancies in which dysregulation of programmed cell death has been observed.^{14,15} As a first step to investigate whether ALG-2 expression is changed in cancer cells we raised antibodies against this protein and tested the applicability of them for immunohistochemical analysis and hence for diagnostic purposes. The two ALG-2 antibodies generated proved to be specific for their target as tested by Western blot analysis in contrast to three commercially available antibodies. One of the newly generated antibodies was tested and found suitable for immunohistochemical analysis. By screening a commercial tissue array we found that ALG-2 is highly expressed in several human cancers, among these a hepatocellular carcinoma,

compared to adjacent normal tissue. Biochemical investigations of rat liver tissues confirmed our findings that ALG-2 expression is markedly increased in cancerous tissues, and by immunohistochemical analysis of four different lung tissue microarrays we found that ALG-2 expression is elevated in both SCLC and non-SCLC.

So far, no molecular mechanism of ALG-2 function in cancer has been described. One approach to get insight into pathways in which ALG-2 could be a player is to identify cancer relevant target proteins and study their function. XP95, the *Xenopus* orthologue of AIP1, which is the first characterized ALG-2 target protein, was shown to be a target for *Src* kinase and to function in cell-cycle regulation.⁷ AIP1 in a complex with ALG-2 was further shown to interact with the SH3 domain-containing protein expressed in tumorigenic astrocytes (SETA), which was also found to be expressed in human astrocytomas of grade II, III, and IV.¹⁶ Overexpression of the N-terminal SH3-binding domain of SETA sensitized astrocytes to death by UV radiation and it was proposed that the AIP1-ALG-2 complex mediated the observed proapoptotic function.¹⁷ SETA was simultaneously isolated by another group¹⁸ and named Ruk for regulator of ubiquitous kinase. Overexpression of full-length Ruk was shown to be lethal for cultured neurons through inhibition of phosphatidylinositol 3-kinase (PI3K).¹⁸ The copy number of the gene *PI3KCA*, coding for the catalytic subunit of PI3K, has been shown to be increased in squamous cell carcinoma.¹⁹ Hence, up-regulation of ALG-2 in lung cancers may be needed to function through an ALG-2-AIP1-SETA/Ruk complex that hinders the inhibitory effect of Ruk on PI3K, thereby allowing signaling through the PKB/Akt survival pathway downstream of PI3K.²⁰ The human SETA/Ruk orthologue, CIN85, has been found to be involved in down-regulating epidermal growth factor (EGF) receptors in a complex with the Cbl proto-oncogene product and endophilins.^{21,22} AIP1 has also been described as a direct endophilin interaction partner and the overexpressed C-terminal SH3 domain-containing part induced vacuolization.²³ It could be speculated that an interface between AIP1, ALG-2, and CIN85 hinders the ubiquitination of the EGF receptors thereby decreasing internalization of EGF receptors, resulting in an abnormal EGF-mediated proliferation signal. Recently, ALG-2 was shown to interact directly with the apoptosis-stimulating kinase 1 (ASK1),²⁴ a MAPKKK. Both ASK1 and AIP1 bind ALG-2 at their C-terminal^{5,24} where a conserved putative SH3-binding domain, PXXP, is found. Similar to AIP1,⁹ ASK1 did not bind the ALG-2 splice variant.²⁴ Taking into

Figure 5. Analysis of lung cancer tissue microarrays using an anti-ALG-2 antibody B1. All sections were stained with DAB+ (brown) and counterstained with hematoxylin (blue). **A:** ALG-2 staining is seen in type II pneumocytes (arrows) and macrophages (arrowheads) of normal alveolar tissue. **B:** Normal respiratory bronchial epithelia shows both cytoplasmic and nuclear ALG-2 immunostaining whereas adjacent stroma does not stain. **C:** Immunoreactivity of serous glandular epithelia (arrows) and ciliated bronchial epithelia with weak ALG-2 staining (arrow) in normal lung tissue. **D:** Staining of cancer cells in a squamous carcinoma in which the central differentiated area (arrowhead) shows less immunoreactivity. **E:** Strong immunoreactivity in the nucleus (arrowheads) of some cancer cells in a SCLC, whereas other cells show only weak nuclear staining (arrows). **F:** Staining of tumor cells (arrowheads) from an adenocarcinoma surrounded by unstained stromal cells. Scale bars, 20 μm (A, B); 50 μm (C-F).

account the Ca^{2+} -binding properties of ALG-2,⁹ it is plausible that small changes in the intracellular Ca^{2+} concentrations result in altered activity of ALG-2, influencing the Ca^{2+} -dependent interaction of ALG-2 with AIP1 and the Ca^{2+} -binding domain containing proteins, peflin, annexin VII, and annexin XI.

Several articles have been published using the ALG-2 antibody from BD Transduction Laboratories.²⁵⁻²⁷ Based on findings presented herein the protein recognized by this antibody cannot be ALG-2. This antibody recognized a band on Western blots with a size of 24 kd (Figure 1) that is clearly larger than the protein from mouse 3D0

cells recognized by the ALG-2 antibody described by Vito and colleagues² and ALG-2 from human, rat, and mouse recognized by the two antibodies, we have produced (Figures 1, 2, and 4). In addition, recombinant ALG-2, whose identity was verified by mass spectroscopy of trypsin fragments, runs at 22 kd and is able to block the immunoreaction with our antibodies in Western blots but not with the antibody obtained from BD Transduction Laboratories (Figure 1). Furthermore, immunoprecipitation with the BD Transduction Laboratories antibody from Jurkat T cell lysates revealed a protein that is not identical to ALG-2 as identified by mass spectroscopy of tryptic peptides (manuscript in preparation). Recently, it was shown that a protein recognized by the monoclonal ALG-2 antibody from BD Transduction Laboratories is up-regulated in two cases of squamous cell carcinoma as well as one mammary tumor.²⁷ Furthermore, ALG-2 was predominantly found in the nucleus of the cancerous cells.²⁷ The latter observation cannot be confirmed based on our investigations. We show that ALG-2 in several cases is found predominantly in the cytosol. The protein detected by the BD Transduction Laboratory antibody was not found in neither protein extracts nor tissue sections²⁷ from normal tissue samples, whereas we find that ALG-2 is ubiquitously expressed (Figure 2), with a predominant expression in epithelial cells (Figure 5B).

Our finding that ALG-2 expression is elevated in cancerous tissues indicates that ALG-2 may have other functions than the alleged proapoptotic. It still needs to be examined whether up-regulation of ALG-2 in cancerous tissues can be used for prognostic purposes. There are only few biomarkers available for the early detection of lung cancers²⁸ and utilization of the ALG-2 antibody for diagnostic purposes in conjunction with prognostic factors may provide improved means for preneoplastic detection.

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