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Novel adapter protein AP162 connects a sialyl-Le^x-positive mucin with an apoptotic signal transduction pathway

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Glycoproteins modified with a sialyl-Le^x-moiety are important sensors for extracellular signals regulating cellular recognition, adhesion and migration. The transduction pathways and signals mediated by these glycoproteins within the cell are largely unknown. In search of novel glycoproteins modified with sialyl-Le^x-moiety, we screened a human colonic cDNA expression library with a rabbit antiserum produced against sialyl-Le^x-positive mucins. The antiserum detected a new protein, named B2, which was cloned and characterised in detail.

The analysis of the B2 gene revealed a 5.7 kb RNA transcript detectable in all investigated tissues and a complete coding sequence of 2778 bp. The B2 protein exhibited two putative PH (pleckstrin homology) domains and a leucine zipper motif but no homology to any known proteins.

Monospecific antibodies against the B2-protein precipitated from the solubilised membrane fraction of the colon carcinoma cell line LS 174T a protein with an apparent $M_r = 162$ kDa and, additionally, a mucin-like glycoprotein with an apparent $M_r = 220$ kDa. Protein fractionation on a CsCl gradient, Western blots and sandwich ELISA showed that the 220 kDa mucin carries the sialyl-Le^x moiety and is tightly bound to the 162 kDa protein.

The expression of the recombinant B2-protein enhanced staurosporine-induced apoptosis in epithelial cancer cell lines.

These data indicate that B2 is a novel, ubiquitously expressed protein with a putative adapter function. The protein has been named AP162 (adapter protein 162). In colon carcinoma cells B2-protein is tightly associated with a sialyl-Le^x-positive mucin and has a potential for involvement in sialyl-Le^x-mediated transduction of apoptotic signals.

Keywords: **pleckstrin homology (PH) domain, PIP-signaling, sialyl-Le^x, colon carcinoma**

Abbreviations: **DTT: Dithiothreitol; SDS: sodium dodecylsulfate, RIPA-buffer: 40 mM sodium phosphate, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 25 μg/ml leupeptin, 109 μg/ml phenyl methyl sulphonyl fluoride (PMSF), 10 mM benzamidine, 10 μg/ml aprotinin; Gua·HCl: guanidinium hydrochloride; PtdIns(3,4,5)P_3: phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4)P_2: phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2: phosphatidylinositol 4,5-bisphosphate; PtdIns(1,3,4,5)P_4: phosphatidylinositol 1,3,4,5-tetrakisphosphate; PI3-K: phosphatidylinositide 3-kinase.**

Introduction

The tetrasaccharide sialyl-Le^x is a terminal structure present on glycolipids as well as on *N*-linked and *O*-linked glycans of plasma membrane glycoproteins of normal and malignant cells [1,2]. On neutrophils, monocytes and resting T cells sialyl-Le^x functions as the ligand for the endothelial leukocyte adhesion molecule E-selectin as well as for the platelet activation dependent membrane protein P-selectin [3].

Several molecules carrying sialyl-Le^x and potentially involved in binding to extracellular receptors have been characterised. On neutrophils the sialy-Le^x moiety has been detected on L-selectin [4] and the carcinoembryonic antigen-like surface molecules NCA-162 and NCA-90 [5]. Another group of sialyl-Le^x-carrying glycoproteins involved in cell adhesion to selectins are the mucin-like molecules PSGL-1, MadCAM and

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GlyCAM on lymphatic cells and CD34 on endothelial cells [3,6]. The mucin molecules have a long rod-like structure with repetetive highly glycosylated regions; this multivalent presentation increases the usually low [7] binding avidity of sialyl-Le^x to the cell surface lectins by several orders of magnitude [8]. Although the role of the sialyl-Le^x molecules in these interaction is well established, little is known how the signal received by these glycoproteins is further processed. This is of particular interest since the discovery that sialyl-Le^x is a carcinoma-associated antigen. Tumor cells have been shown to be enriched with sialyl-Le^x containing glycoproteins and glycolipids [9–12]. In these cells sialyl-Le^x has been detected on the carcinoembryonic antigen [13], on lysosomal membrane glycoproteins lamp-1 and lamp-2 [14] as well as on two mucins MUC1 [15] and MUC2 [16], of which MUC2 is the major mucin in human colon. The overexpression of sialyl-Le^x in colon carcinoma cells was suggested to increase the interaction of blood-borne tumor cells with endothelial adhesion molecules. For example, surface molecule lamp-1 has been shown to effectively present sialyl-Le^x to E-selectin and to mediate tumor cell adhesion to endothelial cells [14]. Several studies demonstrated *in vitro* that the binding of colon cancer cells to selectins expressed on human umbilical vein endothelial cells correlates with the expression of sialyl-Le^x antigen on tumor cells and led to the hypothesis that colorectal cancer cells may use sialyl-Le^x antigen to bind E-selectin in the first step of extravasation [14,17]. Indeed, the amounts of sialyl-Le^x antigen in the metastatic tissue were found to be higher than in the primary tumor [18], and the strong expression of the antigen in tumor tissue was identified as a negative prognostic factor [19,20].

We searched for novel sialyl-Le^x-positive glycoproteins present in the human colon carcinoma. In the present work we report the identification of a sialyl-Le^x-positive membrane mucin tightly associated to a hitherto unknown protein, designated AP162. The detailed analysis of the molecular structure and function of the AP162 protein suggests that it may be involved in transduction of apoptotic signals.

Materials and methods

Human tumor tissue

Tissue from eight colorectal tumours in the Dukes stage C or D was collected immediately after operation and frozen. For mucin preparation about 1 g of each tumor was taken and the pooled tissues were homogenized and further processed. For RNA isolation tissue from three additional tumors was collected and processed immediately after operation.

Production of a rabbit antiserum directed against reduced sialyl-Le^x-positive mucins

Tissue from eight human colorectal carcinomas with the total weight of 8 g was homogenized and used for isolation of mucins. Sialyl-Le^x-positive mucins were purified on three CsCl density gradients as described previously [15]. The obtained purified mucins were reduced with DTT [10 mM, at 60°C for 10 min), acetylated with iodoacetamide (25 mM, at room temperature for 5 h in the dark), dialysed against water, lyophilised and suspended in complete Freund's adjuvant. 100 μg of the purified mucins were intradermally injected into a rabbit. Six injections of the same mucin preparation in incomplete Freund's adjuvant followed in two week intervals. After 15 weeks, when the titer against the reduced carcinoma-associated mucins ceased to increase, the rabbit was killed and bled. Aliquoted serum was stored at −20°C and used for ELISA, screening and for purification of immunoglobulins.

Direct ELISA assay

Native or reduced and acetylated mucin, purified from normal colon mucosa or colon cancer tissue were dissolved in a coating buffer at a concentration of 1 μg mucin protein/ml and adsorbed to a microtiter plate as described previously [15]. The rabbit antiserum was diluted 1:10^4; the purified IgG was applied at a concentration of 100 ng/ml. The detection of bound IgG followed with peroxidase-conjugated goat anti-rabbit immunoglobulins as described [15] and evaluation in an ELISA reader MR5000 (Dynatech, Denkendorf, Germany).

Screening of a human colon cDNA expression library

A human colon λgt11 expression cDNA library (Clontech, Palo Alto, CA) was screened with the obtained rabbit antiserum produced against colon carcinoma-associated mucins. A frequently represented phage clone B2 was recloned and the insert, named B2-gene fragment, was sequenced. Other fragments of the B2 gene (clones B2F and B2E[1]) were found after rescreening of a custom made λZAPII library (Stratagene) of colon carcinoma cell line 5583S, with a PCR-based method as decribed previously [21]. The primers used for the PCR screening reaction were: *forward primer*: GGG CAG CCT CTG GAA CTC AAG; *reverse primer*: GGA AGG CCG GTA GAA GTC ATC CTC TGA GAT. Following PCR conditions were used: 94°C, 30 sec; 63°C, 1 min; 72°C, 45 sec, for 35 cycles.

DNA sequencing and sequence analysis

Radioactive sequencing was performed by the dideoxynucleotide chain termination procedure using a T7 Sequencing kit (USB/Amersham, Cleveland, Ohio) as described elsewhere [22]. Alternatively, cycle sequencing with dyedideoxynucleotides (Perkin Elmer, Weiterstadt, Germany) was performed on an automatic sequencer (373A; ABI/Perkin Elmer). Computer sequence analysis was performed using the HUSAR program package provided by the GENIUS server at the German Cancer Research Center (DKFZ), Heidelberg, Germany. Data base searches were performed using FASTA and BLASTN. Protein motif recognition was also performed using the program

[1]The complete sequence of the clone B2E is available in EMBL databank under the accession No. AJ 002220.

ProfileScan against the PROSITE library provided by the ISREC server.

Isolation of RNA and northern blotting

Total RNA was extracted from malignant colonic tissue with guanidinium thiocyanate followed by centrifugation in CsCl as described [22] and from established cell lines with RNAzolB (AGS, Heidelberg, Germany) according to the recommendations of the manufacturer. Messenger RNA was isolated from the total RNA with Dynabeads (Dynal, Hamburg, Germany) according to the recommendations of the manufacturer. Northern blots were carried out according to the standard procedure with 4.5–6 μg of mRNA per lane, or in the multiple tissue Northern blot with 2 μg mRNA, isolated from different human tissues (Clontech). The sequences from the untranslated 3′ region (bp 4147 to 4681) of the B2 gene, amplified in the PCR or of the B2-gene fragment (bp 855 to 1266) were ^{32}P-labelled and used as probes.

Fractionation of glycoproteins on a CsCl gradient

For detection of the B2 molecule, the lysate of LS 174T cells was separated on the first CsCl gradient, the sialyl-Le^x-positive fractions were identified in slot blot with the AM-3 antibody as described previously, pooled and fractionated on a second gradient. The fractions of the second gradient were reprobed for the presence of B2 protein and the sialyl-Le^x moiety in slot blot with the corresponding antibodies as described [16].

Affinity-purification of the antibodies against the B2-protein fragment

Monospecific antibodies against the B2-protein fragment were purified from the original aliquoted rabbit antiserum by affinity chromatography. For this purpose the B2-gene fragment was expressed in E. coli strain M15 as a tagged His_6-B2 protein of 33 kDa. This protein was detected by the rabbit antiserum in Western blot. The tagged protein was purified on nickel-agarose, coupled to CNBr-Sepharose and used for affinity-purification of the immunoglobulins specific for the B2-protein fragment. From 3 ml of the aliquoted rabbit antiserum about 50 μg Ig specific for the B2-protein fragment were obtained and used for all subsequent experiments. It was termed anti-B2 protein fragment Ig.

Sandwich ELISA

Microtiter plates were coated with the obtained affinity-purified anti-B2 protein fragment immunoglobulins at a concentration of 8 μg/ml as described previously [15]. In parallel wells irrelevant immunoglobulin at the same concentration was applied as the catcher antibody. After washing of the plates and binding of the antigen for 2 h at room temperature, the plates were washed again and the bound antigen was detected with the anti sialyl-Le^x antibody AM-3 [16], conjugated to peroxidase as described [16]. To enhance sensitivity, the BLAST system (Dupont, Bad Homburg, Germany) was used. The background extinction obtained with the irrelevant catcher antibody was automatically substracted by the ELISA reader.

Stable transfection with B2-plasmid

LS 174T cells or MCF-7 cells were stably transfected with pcDNA B2c plasmid or an empty vector by means of the calcium phosphate precipitation method (5Prime-3 Prime Inc., Boulder, Co, USA) and selected in medium supplemented with 0.8 mg/ml G418. The expression of the recombinant B2-protein was monitored in Western blot.

Isolation of the plasma membrane fraction

5×10^7 LS 174T cells were homogenized in 5 ml PBS by ultrasonication and the homogenate was centrifuged at $600 \times$ g for 10 min. The supernatant, containing a mixture of cellular membranes and the cytosol was centrifuged at $100\,000 \times$ g for 1 h. The pellet was solubilised in RIPA buffer (final volume 5 ml each fraction), reduced with DTT (10 mM, 10 min at 60°C), acetylated with iodoacetamide (25 mM) for 5 h at room temperature in the dark, dialysed against PBS and used for immunoprecipitation.

Immunoprecipitation and western blotting

For immunoprecipitation 2 ml of the plasma membrane solubilisate were incubated with 3 μg of the affinity-purified anti-B2-protein fragment IgG for 2 h at 4°C. The immune complexes were precipitated with proteinA-Sepharose, washed three times with 0.5 M lithiumchloride in PBS containing 0.1% Tween, followed by one wash with PBS and water, boiled in sample buffer, electrophoresed on a 3–10% Gel and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with affinitypurified anti-B2-protein fragment immunoglobulins. Then a peroxidase-conjugated second antibody and a chemoluminescence detection with Supersignal (Pierce, Oud Beijerland, The Netherlands) were applied. Detection of sialyl-Le^x antigen was performed with the monoclonal antibody AM-3 as described [16]. The anti-CEA antibody (clone II-7, IgG_1 form DAKO, Hamburg, Germany) anti-CD43 antibody (cloneDF-T1, IgG from DAKO) and anti-CD34 (clone QBEND/10 from Miltenyi Biotec, Gladbach, Germany), anti-PARP antibody (C2-10, Pharmingen, Heidelberg, Germany) rabbit anti-caspase3 antiserum (Pharmingen), anti-phospho Akt antibody (Ser 473, Cell Signaling, Frankfurt, Germany) were used at dilutions recommended by the manufacturer.

Induction and monitoring of apoptosis

LS 174T or MCF-7 cells were seeded 24 h prior to the experiment. Then they were either immediately treated at a given concentration by staurosporine for 16 h or transfected with the

B2-plasmid, incubated for 24 h and then treated with staurosporine at the indicated concentration for another 16 h. Then adherent and floating cells were pooled and lysed with RIPA buffer and PARP cleavage and the caspase 3 fragmentation as a measure of apoptosis were monitored in Western blotting.

Alternatively, DNA fragmentation was detected by the Cell Death Detection ELISA Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. Stable transfected LS 174T cells were treated for 16 h with the indicated concentration of staurosporine. After treatment, adherent cells were trypsinized and pooled with the floating cells. For each sample the same number of total cells was taken and lysed in incubation buffer provided in the kit. The enrichment of fragmented DNA in treated samples relative to the non-treated ones (which were taken as 100%) was determined in ELISA. The experiment was performed three times using triplicates for each drug concentration.

Results

A rabbit antiserum produced against sialyl-Le^x-positive colon carcinoma mucins detects a B2-protein fragment in a cDNA expression bank

An antiserum was produced in a rabbit with sialyl-Le^x—positive mucins purified from human colon carcinoma and used for screening of a colonic cDNA expression library.

The clone B2 obtained by screening of the library and further clones B2F and B2E obtained by rescreening of the library by a PCR-based method had partly overlapping sequences and a common open reading frame of 2778 bp. This sequence was more than 99% homologous to a published unclassified gene No. 356 isolated from a human brain cDNA library (accession number AB 002354 in the EMBL gene bank) [23]. There were 6 single nucleotide differences between B2 versus gene 356 at the positions 78 (A v. G); 187 (C v. T); 282 (G v. C); 462 (T v. C); 601 (G v. A); 3052 (T v. C). Furthermore, in the 5′-nontranslated region a sequence of 124 nucleotides was present in the gene 356 but not in B2. All the differences in the coding region were silent, i.e. the deduced protein sequence was 100% identical (Figure 1A). The sequence is available under the accession number AJ 002220 in the EMBL databank.

Molecular structure of B2

The 2778 bp open reading frame codes for a protein with a predicted M_r of 103 198.5 and pI of 5.73. The aminoacid sequence reveals two PH domains, characteristic for proteins involved in phosphatidylinositolphosphate-mediated signal transduction, and a leucine zipper motif (Figure 1B) but no additional homology to any known protein. The hydropathy plot indicated several potential hydrophobic domains which did not yield a transmembrane protein model (Figure 1C), suggesting that the protein is soluble.

To test the tissue-expression of B2-mRNA, a multiple organ blot and a breast and a colon carcinoma cell lines were used. The

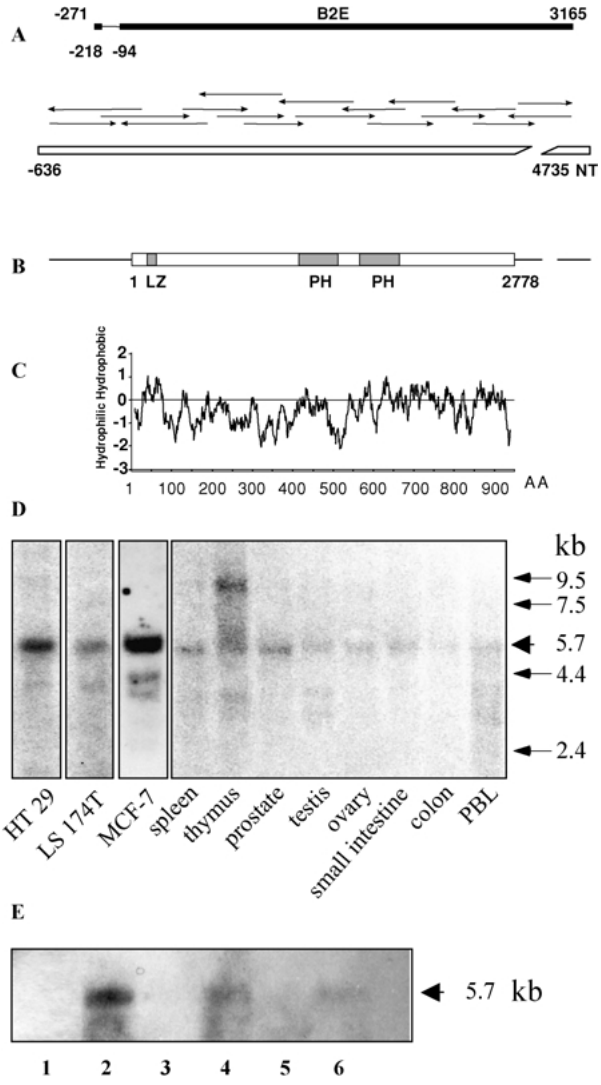

Figure 1. (A) The cDNA clone B2E with the complete coding sequence and the sequencing directions of the B2-gene. The published unclassified gene sequence No. 356, encompassing the nontranslated regions of the B2 gene is shown below. The nontranslated fragment present in the gene No. 356 but absent in the B2 sequence is left out in A. (B) Identified domains in the B2 molecule: leucine zipper (LZ) and PH domains (PH). (C) Hydropathy plot of the B2 protein. (D) Northern blot of mRNA (4.5 μg per lane) isolated from colorectal carcinoma cell lines HT 29 and LS 174T and from mammary carcinoma cells MCF-7 and of mRNA from different human tissues (2 μg per lane). (E) Nothern blot of total RNA (20 μg per lane 1, 3, 5) or mRNA (6 μg per lane 2, 4, 6) isolated from human colorectal carcinoma tissue. Probe: radioactively labeled B2-gene fragment. Molecular weights, given in kb, were determined by running in parallel a RNA standard and staining the gel with ethidium bromide prior to blotting.

main transcript of 5.7 kb in length was detectable in all tissues investigated, with the highest intensity in the thymus, prostate, spleen and peripheral blood lympocytes (PBL) (Figure 1D). This band was also predominant in the breast carcinoma cell

line MCF-7, in the colon carcinoma cell lines LS 174T and HT 29 and in colon carcinoma tissues (Figure 1E). In the thymus a second band at 8.7 kb was detectable. In the spleen, thymus and in the testis two smaller transcripts at 3.8 kb and 3.2 kb were also detectable (Figure 1D). The nontranslated sequence from the 3′ region of the gene No. 356 (bp 4147 to 4681) used as a probe in tissue Northern blot gave the same main band at 5.7 kb (not shown) indicating that the obtained clones and the gene No. 356 have a common 5.7 kb transcript. The nature of the additional bands prominent in the thymus and possibly due to the alternative splicing was not further investigated.

Endogenous as well as the recombinant B2 protein bind tightly to a sialyl-Le^x-positive mucin

To obtain more information on the character of the B2 molecule, the total RIPA lysate of LS 174T cells was reduced with DTT, acetylated with iodacetamide and fractionated on a CsCl gradient containing 4 M Gua·HCl. The sialyl-Le^x-positive fractions were collected and purified on a second CsCl gradient in the presence of 4 M Gua·HCl. In the second gradient, the sialyl-Le^x-positive fractions as well as B2-positive fractions migrated in the same peak with a maximum at a density of 1.37 g/ml, typical for mucins. This result suggested that the B2-protein is associated with a sialyl-Le^x-positive mucin and therefore migrates in the high density peak (Figure 2A).

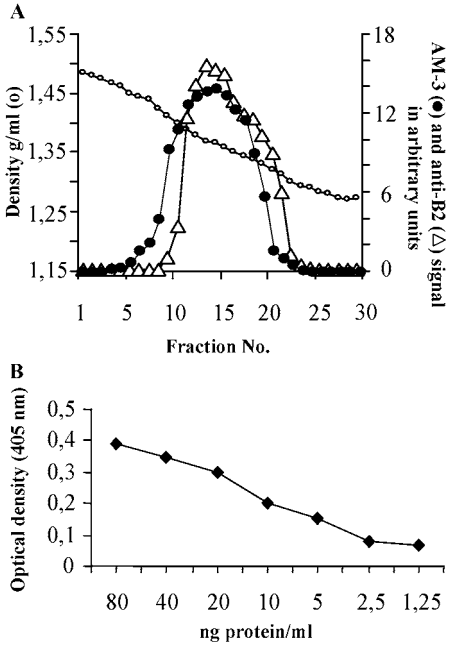

Figure 2. (A) The second CsCl gradient of the reduced lysate of LS 174T cells. Distribution of sialyl-Le^x-positive antigens (dots) and the B2-protein (triangles) detected with anti-B2 protein fragment Ig or AM-3 antibody. (B) Sandwich ELISA of reduced and acetylated cell lysate of the LS 174T colon carcinoma cell line. The monospecific anti-B2-protein-fragment Ig was used as catcher and the peroxidase-labelled anti-sialyl-Le^x-antibody AM-3 as tracer.

Furthermore, a sandwich ELISA with the anti-B2 protein fragment Ig as the catcher, the reduced and acetylated LS 174T cell lysate as antigen source and the AM-3-peroxidase conjugate as tracer, showed that the lysate contained molecular species comprising sialyl-Le^x-epitope and the B2-protein fragment. They supported the notion that the B2-protein is complexed with a mucin molecule which carries sialyl-Le^x moieties (Figure 2B).

The presence of a complex consisting of a mucin and the B2-protein was confirmed by immunoprecipitation. The plasma membrane fraction of LS 174T cells was solubilised and incubated with a monospecific anti-B2 protein fragment antibody. After electrophoresis and blotting, the immunoprecipitate was probed with the anti-B2 antibody or the anti-sialyl-Le^x antibody AM-3. The anti-B2 antibody reacted with a protein with an apparent $M_r = 162$ kDa. The anti-sialyl-Le^x antibody detected in the precipitate a broad band, characteristic for highly glycosylated glycoproteins, with an apparent $M_r = 220$ kDa (Figure 3A). The antibodies against the sialyl-Le^x positive molecules CD43 (leukosialin), CEA and CD45 did not react with the 220 kDa band (not shown).

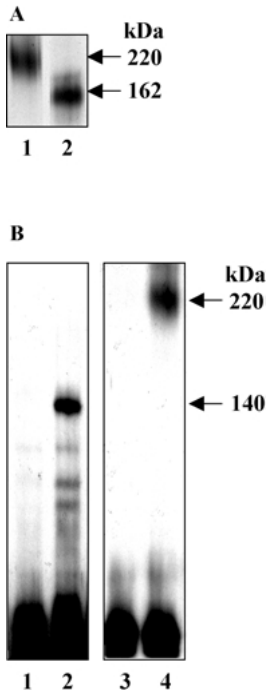

Figure 3. (A) Western blot of the material precipitated with anti-B2-protein IgG from the solubilised plasma membrane fraction of LS 174T cells. The detection followed with anti-sialyl-Le^x antibody AM-3 (lane 1); or with anti-B2-protein antibody (lane 2). (B) Western blot of the anti-myc antibody precipitates from LS 174T cells stably transfected with B2 plasmid (lane 2, 4) or mock-transfectants (lane 1, 3). The detection followed with anti-myc antibody (lane 1, 2) or with the anti-sialyl-Le^x antibody AM-3 (lane 3, 4). There was a difference of 22 kDa between the endogenous ($M_r = 162$ kDa, A) and the recombinant ($M_r = 140$ kDa, B) B2-protein.

The 162 kDa protein did not yield smaller fragments after boiling in mercaproethanol or in 5 M urea for 10 min. It was concluded that in the plasma membrane the B2 protein ($M_r = 162$ kDa) is present in a complex with a sialyl-Le^x-positive mucin glycoprotein ($M_r = 220$ kDa). The two components were bound tightly enough to comigrate in the CsCl gradient and to coprecipitate from the cell lysates but were separated after boiling in sample buffer and SDS-PAGE.

To proof if the recombinant B2-protein was binding to the sialyl-Le^x-positive mucin molecule, the immunoprecipitation was carried out after stable transfection of LS 174T cells with a plasmid coding for the full length B2-protein with a myc tag. The immunoprecipitation with the anti-myc antibody and the immunoblotting of the precipitate showed that the recombinant protein coprecipitated with the 220 kDa sialyl-Le^x-positive protein, as did the endogenous one (Figure 3A and B). The recombinant protein had, however, a lower molecular weight ($M_r = 140$ kDa) than the endogenous protein, thus suggesting that the latter is posttranslationally modified in a manner not affecting the binding to the mucin glycoprotein.

The recombinant B2-protein enhances staurosporine-induced apoptosis

The PH domains present in the B2-protein may mediate interaction with PI(3,4)P_2 as well as with proteins comprising PH domains. Since some kinases exhibit PH domains, the effect of staurosporine, an inhibitor of protein kinases, on cells transfected with the recombinant B2-protein and on mock-transfectants were investigated.

Staurosporine triggers apoptosis in colon carcinoma cells. It induced stronger apoptosis in LS 174T cells stably transfected with the B2 protein than in mock-transfectants as seen by the fragmentation of DNA (Figure 4A) as well as of PARP and caspase 3 (Figure 4B). The same enhancement of staurosporine-induced PARP fragmentation was observed in breast carcinoma cells MCF-7 after transient transfection with B2 protein (Figure 5). The specific inhibitor of protein kinase C, bis-indolyl maleimide did not have an effect on the transfected nor on the mock-transfected cells, indicating that the PKC was not involved in the staurosporine-induced cell death. The treatment with staurosporine did not alter the intracellular level of cAMP (data not shown) thus confirming that neither PKC nor adenylate cyclase-dependent signal transduction pathway was activated.

The recombinant B2-protein does not inhibit Akt-kinase activation by staurosporine

A preeminent kinase comprising a PH domain is Akt, also called PKB-kinase. Its phosphorylation and activation occurs after recruitment of Akt to the membrane by binding to PI(3,4)P_2 through PH domains and frequently leads to suppression of apoptosis [24].

Other proteins with the PH domain could potentially antagonize Akt by inhibiting its translocation to the plasma membrane [25]. To test if the recombinant B2-protein competes for PI(3,4)P_2 with Akt and thus inhibits its activation, we investigated the activation status of Akt after treatment of B2-transfected or mock-transfected MCF-7 cells with staurosporine. While the staurosporine-induced apoptosis measured by PARP cleavage was stronger in the transfectants than in the mock-transfectants, the concomitant strong activation of Akt was similar in both groups of treated cells (Figure 5).

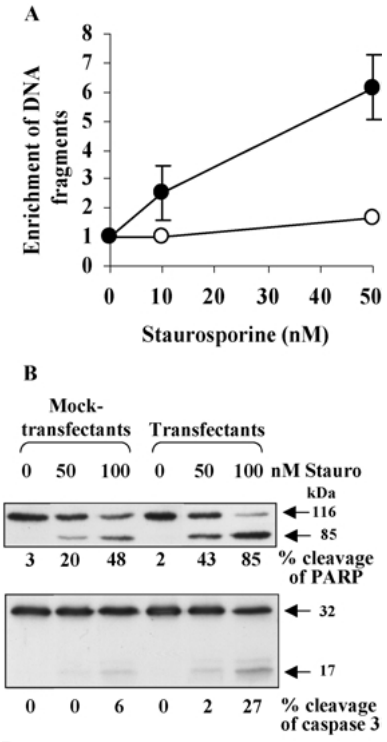

Figure 4. Effects of staurosporine treatment for 16 h on LS 174T cells stably transfected with the recombinant B2-protein versus mock-transfected cells. (A) Higher enrichment of the fragmented DNA in the B2-transfectants (black dots) than in the mock-transfectants (white dots) after treatment. Means of three experiments ± SD. (B) Staurosporine-triggered fragmentation of PARP and of the caspase 3 protein is enhanced in the B2-transfectants.

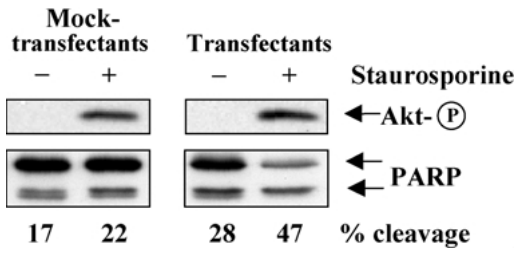

Figure 5. MCF-7 cells transiently transfected with B2-protein or with the empty vector (mock transfectants) and treated with staurosporine (200 nM, 16 h) phosphorylate Akt to the same extent while the cleavage of PARP is higher in the transfectants than in the mock transfectants.

This showed that staurosporine induces strong activation of Akt and, simultaneusly, apoptosis in MCF-7 cells. It demonstrated further, that the recombinant B2 protein does not interfere with Akt activation. In view of its molecular weight and the putative function as an adaptor, the protein has been named AP162.

Discussion

In the present work a novel protein, named AP162, with an apparent M_r of 162 kDa was detected in several human tissues including colon and colon carcinoma and was characterized on the molecular level. In colon carcinoma cells and tissues, as well as in breast carcinoma cells it occurs in a complex with a sialyl-Le^x-positive glycoprotein. The structure, the cellular distribution and the functional assays suggest that the protein AP162 may function as an intracellular adaptor modulating apoptotic signals.

The membrane-located glycoprotein, with an apparent M_r = 220 kDa, migrated in a CsCl gradient at a density of 1.37 g/ml and yielded in Western blot a broad diffuse band, both indicative of mucin-type glycoprotein. Its apparent molecular weight in SDS-PAGE is different from MUC1 and MUC2, the two mucins known to carry sialyl-Le^x in colon and colon carcinoma [15,16]. Several molecules of a similar molecular weight, e.g. CD43 (leukosialin, M_r = 150–300 kDa [26], CEA (180 kDa) and CD45 (200 kDa) were excluded by Western blotting. Other sialyl-Le^x-positive molecules like L-selectin [27], lamp-1 [14] and the mucin molecules GlycaM-1, CD34 and PSGL-1 have the apparent molecular weights between 50 and 120 kDa. It appears, therefore, that it is a hitherto not described, sialyl-Le^x-positive, mucin molecule. Cloning of the apoprotein part of the molecule is necessary to analyse its potential relationship to the already described sialyl-Le^x-positive mucins.

The AP162-mucin complex was found in the solubilisates of the membrane fraction, suggesting that at least one component has a hydrophobic, possibly transmembrane domain. Since no transmembrane domain has been found in the aminoacid sequence of AP162-protein, the data are compatible with the hypothesis that AP162 binds to the cytoplasmic tail of the 220 kDa mucin. The two components dissociated only after reduction and boiling in SDS-containing buffer, indicating a strong hydrophobic interaction between the two molecules. Whether the leucine zipper motif is involved in the AP162-mucin heterodimer formation is not clear at present.

The open reading frame of the main AP162 transcript has 926 aminoacids and corresponds to a calculated M_r of 103 198.5 Da. The discrepancy between the experimental (162 kDa) and the calculated value may be due to the presence of high content of acidic aminoacids, reflected by the low pI of 5.73 and to posttranslational modifications.

The obtained cDNA sequence was homologous to the sequence of a published unclassified gene No. 356. Both genes have in common a transcript of 5.7 kb of which only 2.778 kb are translated. Six single nucleotides and a sequence of 124 nucleotides in the nontranslated region were different between B2 and the gene No. 356. These differences, which may be due to the different sources of the DNA used and to the gene polymorphism did not affect the aminoacid sequence which was identical.

Of particular interest is the presence of two PH (pleckstrin homology) domains, (92 and 95 aminoacids in length) as well as of the leucine zipper within the AP162 aminoacid sequence. These domains imply that AP162 interacts with other proteins and may be involved in signal transduction. In fact, the PH domains are found in many molecules involved in signalling and controlling such diverse processes like growth, survival and cell adhesion [28]. Their about 100 aminoacids-long sequence can mediate lipid-protein as well as protein-protein interactions. The molecules comprising this motif include small GTP binding proteins, protein kinases, subtypes of phospholipase C, cytoskeletal proteins, kinase substrates and adapter proteins.

The PH domain has recently been shown to function as an adapter structure which, similarly to SH2 domains, anchors cytosolic proteins in the membrane by binding to membrane-bound phosphatidylinositolphosphates [29]. Individual PH domains have specificity for distinct phosphoinositides. For example, the key enzyme in the PI3-kinase signaling pathway, Akt, binds via its PH domain to PI(3,4)P_2 [30,31]. This binding stimulates the Akt activity and, also, its phosphorylation by the appropriate Akt-kinase. The PH domain of the regulatory molecule cytohesin 1 binds to PtdIns(1,3,4,5)P_4 [32] and that of the general receptor for phosphoinositides, Grp1, to PtdIns (3,4,5)P_3 [33]. Through this selective binding the PH domains appear to confer specificity to downstream target phosphorylation in response to extracellular stimuli.

The signalling processes triggered by sialyl-Le^x-carrying molecules in leukocytes or in epithelial cells are only fragmentarily understood [34]. In neutrophils the signal is presumably transmitted through G-proteins, which activate integrins on the surface of leukocytes, establishing a firm attachment to endothelial cells [3,35] while in T cells CD43 (leukosialin) has a cytoplasmic domain which is constitutively phosphorylated and is thought to be engaged in the transmembrane signalling [36–38]. In epithelial cells the mucin MUC1 has been identified as a potential signal transduction molecule [39]. Its cytoplasmic tail contains phosphotyrosines which interact with the adapter protein Grb2 [40]. The probing of the B2-mucin complex with anti-phosphotyrosine antibodies in protein blot did not, however, reveal any phosphorylated groups in either molecule (not shown).

Recent data indicate that adapter proteins containing PH domains may also interact with membrane glycoproteins. For example, the cytoplasmic regulatory molecule with a PH domain, cytohesin 1, binds to the cytoplasmic domain of the integrin ß2-chain. The intracellular signals received via the PH domain of cytohesin 1 appear to regulate αLß2 integrin-mediated cell adhesion [41]. These molecules frequently receive extracellular

signals via the carbohydrate moieties and transmit them into the cell and it is possible that AP162-mucin complex is involved in such a function.

The present data clearly indicate that AP162 is a modulator of the apoptotic signal triggered by staurosporine. This signal—which is not involving PKC—induces apoptosis, which is enhanced by AP162. The induction of apoptosis by staurosporine was associated with the activation of the usually antiapoptotic activity of Akt, in agreement with a recent report [42]. These data are compatible with the hypothesis that AP162 directly or indirectly interacts with a kinase—different from Akt—which is constitutively inhibiting apoptosis. It will be interesting to investigate if extracellular signals sensed by sialyl-Le^x are involved in this modulation.

In conclusion, in the present work a complex of a sialyl-Le^x-positive mucin molecule and a novel protein AP162 was identified and the AP162 protein was analysed in detail. The functional role of the AP162-mucin complex, in particular the involvement in modulation of apoptotic signals in colon and colon carcinoma warrants further study.

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