Introduction

Complement DAF\(^3\) or CD55 is a member of a family of proteins involved in the inactivation of complement. CD55 inhibits the complement cascade by inactivating C3 convertases and preventing C3b deposition on cell membranes. C3b is a powerful stimulus of phagocytosis by antigen-presenting cells and also initiates the formation of the membrane attack complex (1). CD46 and CD59 are two other membrane-associated complement regulators that also inhibit C3b deposition and association of the membrane attack complex [reviewed by Liszewski et al. (2)]. All three of these proteins have been shown to be overexpressed by a range of solid tumors and have been implicated in the limited success of antibody-mediated lysis of tumor cells (3). However, their role in inhibition of antigen processing of tumors is unknown. The three complement regulatory proteins share a similar structure comprising four SCR domains. CD55 and CD59 are GPI anchored to the cell membrane, whereas CD46 has a classical transmembrane domain (reviewed in Ref. 4). Significantly, they have all been implicated in signal transduction in T cells.

The tumor associated antigen 791Tgp72 was characterized by a monoclonal antibody 791T/36, which was raised against an osteosarcoma (791T) cell line (5). The antigen was originally described as a 72 kDa membrane associated glycoprotein (6) that is over-expressed on a range of tumors including colorectal, gastric, ovarian, and osteosarcomas (7, 8). Furthermore over-expression of the antigen con-fers a poor prognosis in colorectal cancer patients (9). The specificity of the antibody has been shown by clinical imaging of over 300 patients, in which lesions as small as 1 cm\(^2\) were detectable in 70% of patients. (10–12). During these imaging studies the majority of patients showed the production of human anti-mouse antibodies (HAMA). A large component of which was directed against the idiotype of 791T/36 (13). The human monoclonal anti-idiotypic antibody, 105AD7, which binds at the combining site of 791T/36 and mimics the antigen was isolated from one of these patients (14). Pre clinical studies have shown that 105AD7 is able to stimulate antibody and T-cell responses to the tumor antigen in both rats and mice (15). Clinical studies with the human anti-idiotype has shown similar results in that patients receiving the vaccine show in vitro proliferation responses to either 105AD7 or tumor cells expressing CD55, elevated serum interleukin 2, and decreases in the CD45 RA:RO ratio indicating activation of both CD4 and CD8 cells (16). Furthermore a neo-adjuvant clinical study demonstrated enhanced tumor killing (17) and infiltration of CD4, CD8 and NK cells within the tumors of immunized patients (18).

In this study, we have purified, characterized, cloned, and sequenced the 791Tgp72 antigen from tumor cells and shown that it is identical in sequence to CD55 (DAF). This molecule is normally expressed by cells to protect them from complement-mediated lysis. Our previous studies have characterized this antigen as being overexpressed by a range of tumors, making it a good target for imaging and proving to be of prognostic significance in colorectal carcinoma (9). The anti-idiotypic antibody 105AD7 acts as a mimic of CD55 and is capable of stimulating T-cell responses in vitro (17). These findings identify CD55 as another normal antigen, along with MAGE, tyrosinase, and gp100 that are expressed by tumors and which can act as targets for immunotherapy [reviewed by van den Eynede and Van der Bruggen (19)].

Materials and Methods

Cells. 791T is an osteosarcoma cell line obtained from the American Type Culture Collection and is grown in RPMI containing 10% FCS. CHO cells were obtained from the European Collection of Cell Cultures and maintained in DMEM, 10% FCS containing essential amino acids (Sigma Chemical Co.).

Monoclonal Antibodies. Monoclonal antibodies 791T/36 (IgG2b anti-791Tgp72; Ref. 5), BRIC 216 (IgG1 anti-SCR 3 of CD55), BRIC 220 (IgG1 anti-SCR 1 of CD55), and BRIC110 (IgG1 anti-SCR 2 of CD55) were used. The BRIC antibodies were obtained from the International Blood Reference Group laboratory (Bristol, UK). 791T/36 was labeled with FITC (14). Monoclonal antibody 1H4 was a kind gift from Dr. Bruce Loveland (Austin Research Center, Melbourne, Australia).

Protein Sequence Analysis. Immunoaffinity-purified 791Tgp72 (10 µg) was subjected to 16 cycles of Edman degradation on an ABI Model 473A automated protein sequencer following SDS-PAGE under standard conditions and electroblotting onto polyvinylidene difluoride membrane (20). Sequence data were compared to all known proteins using the BLASTp algorithm at National Centre for Biotechnology Information (21).

Binding of anti-DAF Antibodies to Purified 791Tgp72. Flexible microtiter plates (Falcon; Becton Dickinson, CA) were coated with either purified 791Tgp72 antigen, 50 ng/well at 37°C, or purified CEA (500 ng/well). Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween)
and blocked with BSA (1%) for 1 h at room temperature. Plates were washed three times, and primary antibodies [anti-CEA (365), 791T/36, and anti-CD55 antibodies, BRIC 220, BRIC 216, and BRIC 110] that recognize SCR domains 1, 2, and 3 respectively] were added at 500 ng/well for 1 h at room temperature. After three washes in PBS-Tween, plates were incubated with rabbit anti-mouse horseradish peroxidase (Dako, Denmark) at 1:1000 for an additional 1 h before developing with 2,2'-azinobis(3-ethylbenzthiazoline sulphonic acid) (ABTS). Plates were read at 405 nm.

**Double Determinant ELISA.** Plates were coated with anti-CD55 antibodies 220 (SCR 1), 110 (SCR 2), and 216 (SCR 3) and left at 4°C overnight. The plates were washed three times with PBS-Tween and blocked with BSA (1%) for 1 h at room temperature. The plates were washed three times, and then purified CD55 antigen (25ng) was added. After 1 h at room temperature, the plates were washed three times, and biotinylated 791T/36 (500ng per well) was added for 1 h at room temperature. Plates washed three times, and streptavidin-horseradish peroxidase (DAKO) diluted 1:1000 was added for an additional 1 h. After an additional six washes, the plates were developed with ABTS (Sigma, Poole, United Kingdom), and absorbance was read at 405 nm.

**DNA Cloning and Sequencing.** Total cellular RNA was isolated using RNasol B (Life Technologies, Ltd., Paisley, United Kingdom) from 791T cells (4 × 10^5). First-strand cDNA synthesis was carried out using Ready-To-Go First-Strand kit (Pharmacia Biotech, Little Chalfont, United Kingdom). Primers based on the NH2-terminal protein sequence of the purified antigen [5'-GACTGGGCATCGCCGACATG-3' (PEP5)] and the full-length CD55 cDNA [accession no. M15799; 5'-ATGTGATTCCAGGACTGCC-3' (FL3)]. These were used to generate products by reverse transcription-PCR on duplicate RNA samples from 791T cells. PCR was carried out on first-strand cDNA, and primers used were mixes of the primer sets outlined above. Thirty cycles of amplification at 94°C for 30 s, 55°C for 45 s, 72°C for 90 s, followed by 10 min at 72°C were performed using Deep vent DNA polymerase in a reaction mix containing 2% DMSO (New England Biolabs, Hitchin, United Kingdom). PCR products were cloned into modified pBluescript SK-vector. Sequencing was carried out on an ABI Model 373 automated DNA sequencing using M13 Universal and Reverse sequencing primers as well as PEP5 and a second internal primer SU25 (5'-AGCTGGCAGGTGCCAAC-3'). Data were analyzed by BLASTn at the National Centre for Biotechnology Information (21). Full-length clones were subcloned into the EcoRI site of pCR 3.1 (Invitrogen) for transfection into eukaryotic cells.

**Expression of CD55.** CHO cells grown in six-well Costar plates to 50–60% confluence were transfected with pCR 3.1 containing full-length DAF cDNA using Lipofectamine (Life Technologies, Inc.; 2 μg DNA, 9 μl lipofectamine) for 12–16 h at 37°C, prior to changing the growth medium and according to the manufacturer's recommendations. Protein expression was measured 48 h after transfection by indirect immunofluorescence staining with 791T/36 antibody. Cells were analyzed by flow cytometry (FACS Scan; Becton Dickinson, Sunnyvale, CA).

**Western Blotting.** Cells were trypsinized, washed in PBS, and solubilized in lysis buffer [1% octyl-glucoside (Sigma, Poole, United Kingdom), 20 mM Tris-HCl (pH 8.0), and 140 mM NaCl] for 30 min at 4°C. Supernatant was removed after centrifugation at 13 Krpm for 10 min and diluted 1:1 with nonreducing SDS-sample buffer. Samples were electrophoresed on 8% SDS-PAGE and Western blotted onto Hybond-N membrane (Amersham-Pharmacia, Little Chalfont, United Kingdom). The blot was washed in PBS and then blocked in 1% BSA in PBS-Tween (0.1%) for 1 h at room temperature. Primary antibody was added in wash buffer [PBS TWEEN (0.1%)] for 1 h at room temperature. The blot was washed twice, and HRP-anti mouse (1/1000 in PBS) was added for 1 h at room temperature. The blot was finally washed twice and visualized using ECL reagent (Amersham-Pharmacia UK).

**Results**

The antigen 791Tgp72 was purified to near homogeneity on a cross-linked protein-A-791T/36 affinity column. This procedure and the use of 1% octyl-gluco side greatly improved the efficiency of the purification and allowed the resolution of two proteins with apparent molecular masses of 66 and 72 kDa from 791T cells. The purity of eluted fractions was assessed by SDS-PAGE and silver staining (Fig.

![Fig. 1. SDS-PAGE of affinity eluates including NH2-terminal amino acid sequence.](image)

![Fig. 2. Western blot of cell-associated and purified antigen using anti-CD55 antibodies.](image)

![Fig. 3. Binding of anti-CD55 antibodies to purified 791Tgp72 and CEA. ELISA plates were coated with affinity-purified 791Tgp72 or CEA. Monoclonal antibodies to SCR domains 1, 2, and 3 of CD55 (220, 110, and 216), anti-791Tgp72 (791T/36), and an anti-CEA monoclonal (365) were used in conjunction with a primer from the 3' untranslated region of the cDNA of CD55 [5'-ATGTGATTCCAGGACTGCC-3' (FL3)]. These were used to generate products by reverse transcription-PCR on duplicate RNA samples from 791T cells. PCR was carried out on first-strand cDNA, and primers used were mixes of the primer sets outlined above. Thirty cycles of amplification at 94°C for 30 s, 55°C for 45 s, 72°C for 90 s, followed by 10 min at 72°C were performed using Deep vent DNA polymerase in a reaction mix containing 2% DMSO (New England Biolabs, Hitchin, United Kingdom). PCR products were cloned into modified pBluescript SK-vector. Sequencing was carried out on an ABI Model 373 automated DNA sequencing using M13 Universal and Reverse sequencing primers as well as PEP5 and a second internal primer SU25 (5'-AGCTGGCAGGTGCCAAC-3'). Data were analyzed by BLASTn at the National Centre for Biotechnology Information (21). Full-length clones were subcloned into the EcoRI site of pCR 3.1 (Invitrogen) for transfection into eukaryotic cells.

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Affinity-purified antigen was separated by SDS-PAGE and Western blotted onto polyvinylidene difluoride membrane. Sixteen cycles of NH$_2$-terminal sequencing were carried out on the 66 and 72 kDa bands, resulting in the following sequence data “DCGLPPDVPNAQ-PALE” for both proteins (Fig. 1, inset). This showed 100% identity with the protein sequence of complement DAF (CD55).

The purified antigen was assessed for its ability to be recognized by anti-CD55 monoclonal antibodies. Both antigen-positive cells and purified antigen were analyzed by Western blotting using anti-CD55 antibodies BRIC 216 and 110 as well as 791T/36. The blot (Fig. 2) shows that both anti-CD55 antibodies and 791T/36 are able to recognize both forms of the antigen on both antigen-positive cells and purified antigen.

Three monoclonal antibodies recognizing different domains of CD55 were used to characterize their recognition of the purified antigen. The antibodies BRIC 220, 110, and 216 recognize SCR domains 1, 2, and 3 of CD55, respectively. These antibodies along with 791T/36 showed significant binding to the purified antigen, whereas the anti-CEA antibody (365) gave background levels of binding to the CD55 antigen. In comparison, the CD55 antibodies did not recognize another GPI-anchored protein (CEA), whereas the anti-CEA monoclonal antibody gave high levels of binding (Fig. 3).

The availability of a range of monoclonal antibodies to the various SCR domains of CD55 allowed us to undertake preliminary epitope mapping studies with 791T/36. Double determinant ELISA assays were carried out whereby one antibody was used to capture the antigen and a second antibody (biotinylated 791T/36) was used to detect the bound antigen, allowing approximate mapping of epitopes (Fig. 4). When two antibodies bind to the same unique region, the detecting antibody fails to give a signal. This is seen when 791T/36 is used as both the capturing and detecting antibody. In contrast, antibodies recognizing distinct epitopes give strong signals, as was observed when either monoclonal antibody 220 (SCR1) or 216 (SCR3) was used as capturing antibodies. However, monoclonal antibody 110 that recognizes SCR2 does appear to hinder the binding of 791T/36 in this assay.

To confirm that 791Tgp72 was CD55 and not a mutant or splice variant expressed by tumor cells, the cDNA encoding the 791Tgp72 protein was cloned and sequenced. Primers based on the NH$_2$-terminal amino acid sequences of both 791Tgp72 and p66 proteins and the published full-length sequence of CD55 were used to PCR CD55-related molecules from the 791T tumor cell line. The 18 clones obtained from several cDNA preparations and multiple PCR clonings were exhaustively sequenced from both ends and by using an internal primer. The sequence obtained from these reactions displayed 100% homology with the CD55 sequence reported previously (accession no. M15799).

Transfection of full-length cDNA of CD55 into CHO cells resulted in binding of 791T/36 antibody to the transfected but not untransfected cells (Fig. 5A). Two transfected cell lines were also assessed for CD55 production by Western blotting (Fig. 5B). The transfected CHO cells produced a band of ~60–65 kDa that was recognized by the anti-CD55 monoclonal antibody 110.
791T/36 antibody. This band was not present in the untransfected CHO cells.

Discussion

Clinical studies identified 791Tg72 as a good target antigen for immunotherapy. To understand the molecular basis for the clinical observation, it was important to characterize the antigen. This study provides evidence that 791Tg72 tumor-associated antigen is CD55. Immunoaffinity purification of the antigen from the osteosarcoma cell line 791T results in the isolation of two predominant bands of apparent molecular masses of \( M_r \) 72,000 and \( M_r \) 66,000. \( \text{NH}_2 \)-terminal protein sequencing of the 66/72 kDa bands showed identity with CD55 or DAF. CD55 was originally purified by Nicholson-Weller from human erythrocytes (22) and was identified as a single chain glycoprotein of 70 kDa by SDS-PAGE. By Western blot analysis, both 791T/36 and two anti-CD55 antibodies were able to recognize both forms of the antigen on cells and as purified protein. The specificity of this was tested using the anti-CD55 monoclonal antibodies, recognizing SCR 1, 2, and 3, binding to purified 791Tgp72 but not another tumor-associated GPI-anchored protein, CEA. This supported the \( \text{NH}_2 \)-terminal amino acid sequence data, demonstrating that monoclonal antibodies recognizing three distinct regions of CD55 showed specific binding to the purified 791Tgp72 antigen. However, the anti-CD55 monoclonal antibodies recognizing SCR domains 1 and 3 were not able to impede the binding of 791T/36 to captured antigen in a double determinant sandwich ELISA, but the antibody 110 that recognizes SCR2 appeared to hinder the binding of 791T/36 to CD55. This may imply that 791T/36 binds at a site that is close to the site recognized by the anti-CD55 monoclonal antibody (BRIC 110).

Cloning and sequencing PCR products generated from primers derived from the \( \text{NH}_2 \)-terminal protein sequence and a primer derived from the published CD55 sequence resulted in nine clones that were homologous to CD55. An additional nine clones generated from primers based on the full-length sequence of CD55 confirmed the identity of the previous sequence data and gave the additional 166 bp of sequence from the 5′ untrated region encoding the leader sequence of CD55. The sequence identity from these clones confirmed that the cDNA encoding the 791Tgp72 antigen is the same as CD55. Expression of this cDNA in antigen-negative CHO cells resulted in binding of 791T/36 monoclonal antibody to the transfected but not the untransfected cells. Furthermore, Western blot analysis of these transfected clones revealed a single 60–65 kDa band in the transfected cells only. This single band of ~63 kDa was reported by Coyne et al. (23) when using full-length CD55 cDNA transfected into CHO cells.

The function of CD55 is to protect cells from complement-mediated lysis. Therefore, CD55 is expressed on all normal cells that are exposed to complement, including blood cells, endothelial cells, and epithelial cells that are exposed to complement. The prevalence of CD55 on normal cells raises the question of why it was possible to successfully image with the monoclonal antibody 791T/36 and stimulate antitumor T-cell responses with an anti-idiotypic vaccine with no normal cell toxicity. The answer may be in the levels of antigen expressed, because 791T/36 binds strongly (105–106 molecules/cell) to osteosarcomas, ovarian, gastric, and colorectal tumors (7), whereas other groups have shown that 104 molecules of CD55 on RBCs is sufficient to protect them from complement-mediated lysis. However, blood samples taken from patients after injection of the radiolabeled 791T/36 showed only background levels of radioactivity on peripheral blood mononuclear cells and RBCs. It could be that the low antigen expression on RBCs only allowed weak monovalent 791T/36 binding. However, in the presence of high antigen density in the tumor, divalent higher affinity binding would be favored, and the antibody would accumulate at the tumor cell surface.

Overexpression of CD55 by tumors may not only protect from the lytic effects of complement but also from the deposition of C3b, which leads to endocytosis by antigen-presenting cells. This may explain why tumors in situ fail to generate an effective immune response, despite the “danger signals” generated by necrotic tissue. It has been demonstrated that activated T cells that have been cross-linked with anti-CD55 monoclonal antibodies can induce T-cell proliferation and signal transduction via \( p56^{\text{IL-2R}} \) and \( p59^{\text{FR-3}} \) (24, 25). This may be related to the recent observation that CD55 is the ligand for the CD97 receptor expressed on activated T cells (26). Furthermore, cross-linking of the related family member CD46 results in down-regulation of interleukin 12 production by antigen-presenting cells, with an associative switch to a Th-2 or humoral immune response. Any antibodies would be effectively inhibited from complement lysis of tumors by the same overexpression of complement regulatory proteins, with the result being immune deviation rather than immune ignorance of the growing tumor. Additionally, overexpression of CD55 by tumor cells may result in the tumor becoming more resistant to NK lysis, as has been demonstrated by overexpression of CD55 on K562 target cells; interestingly, NK cells are the only mononuclear cells that do not express CD55 (27). Many tumors escape T-cell recognition by loss of MHC molecules, making them susceptible to NK killing. Overexpression of CD55, however, may inhibit NK lysis and would be an obvious advantage to the tumor.

Whether tumor cells overexpress CD55 to protect them from complement-mediated lysis or whether the enhanced level is related to an unknown function of this molecule is yet to be elucidated. However, it remains an interesting prospect to use a molecule that tumors overexpress to protect them from immune attack, as a target for a cancer vaccine. We have characterized this antigen as a tumor target based on clinical data in which a mimicking anti-idiotypic has been able to stimulate immune responses to the antigen. This approach is not unique because anti-idiotypic responses to mutant p53 in the mouse (28), CEA (29), and GA733-2 (30) in the human have shown effective T-cell immunity to tumor antigens. These are perhaps not as unusual as immune responses against an antigen whose normal function is to protect cells from immune attack, the dichotomy being that if the cell fails to express the molecule, it is susceptible to complement and NK-mediated lysis, and if it overexpresses the antigen, it may be a target for antigen-specific T cells. Despite the widespread distribution of this antigen, we have experienced no measurable toxicity in >160 patients receiving the 105AD7 anti-idiotypic while monitoring significant increases in T-cell infiltration and NK activity within tumors of immunized patients.

References

Decay Accelerating Factor (CD55): A Target for Cancer Vaccines?

Ian Spendlove, Li Li, James Carmichael, et al.


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