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Properties of the co-chaperone protein p23 erroneously attributed to ALG-2 (apoptosis-linked gene 2)

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Abstract A commercial antibody (clone 22) directed against the apoptosis-linked gene 2 (alg2, pdcd6) encoded protein has been used by several groups. Up-regulated expression of the antigen was observed in primary tumours and in metastatic tissue and also during rat brain ischemia. Furthermore, antigen down-regulation was found in human atherosclerotic plaques. Recently, we found that the clone 22 antibody does not recognise ALG-2. In the present study the antigen of the clone 22 antibody was identified as the heat shock protein 90 (HSP90) co-chaperone protein p23, identical to the cytosolic prostaglandin E2 synthase, by immunoprecipitation followed by tryptic in-gel digests and mass spectrometry of the purified peptides. Moreover, the heterogeneous ribonuclear protein A2/B1 was found to be a part of the p23 co-immunoprecipitated protein complex.

Key words: Heterogeneous ribonuclear protein A2/B1; Prostaglandin E2 synthase; PYK2; Ischemia; Cancer; Atherosclerosis

1. Introduction

In a recent study we found that the clone 22 Becton Dickinson (BD) Transduction Laboratories monoclonal antibody assumed to recognise apoptosis-linked gene 2 (ALG-2) did not detect a protein with the correct size [1]. Since part of the published research on ALG-2 therefore was based on false assumptions, we intended to identify the antigen of the clone 22 antibody. In this paper we have identified the antigen as the heat shock protein 90 (HSP90) co-chaperone p23 that has recently been shown to be the cytosolic prostaglandin (PG) E2 synthase (cPGES), one of the enzymes downstream of the cyclooxygenases (COX-1 and COX-2) [2]. COX-1 and COX-2 deliver the prostanoid precursor PGH_2 for the enzymes, which catalyse the synthesis of thromboxanes, prostacyclines, and PGs. The COXs are functionally coupled to two different types of PGE_2 synthases. COX-1 is coupled to cPGES, whereas COX-2 primarily couples to the membrane bound PGES (mPGES). By their contribution to the synthesis of potent biological mediators, the COXs are known to be involved in several human pathologies including pain, inflammation, fewer, thrombosis, cancer, and neurological disorders such as Alzheimer's and Parkinson's diseases (see [3]).

Based on our finding that the BD clone 22 antibody recognises p23 but not ALG-2, and the previous published papers describing features of the antigen to the clone 22 antibody, we suggest that p23 may play a role in several pathologies including cancer, ischemia and atherosclerosis.

2. Experimental

2.1. Reagents and antibodies

Affinity purified rabbit anti-ALG-2 antibodies were produced in our laboratory as previously described [1]. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulins and goat anti-rabbit immunoglobulins were purchased from DakoCytomation, Glostrup, Denmark. The mouse monoclonal IgG_1 anti-ALG-2, clone 22, the mouse monoclonal IgG_1 anti-p23, clone 16, and the mouse monoclonal IgG_1 anti-Fas/CD95, clone 13, were from BD Transduction Laboratories (BD Biosciences, San Jose, FL, USA). The mouse monoclonal IgG_1 anti-p23, clone JJ3 was kindly provided by David Toft, Mayo Clinic, Rochester, MN, USA. The mouse monoclonal IgG_3 anti-Fas/CD95/APO-1, clone APO-1-3 was a kind gift from Peter Krammer, Deutsches Krebsforschungszentrum, Heidelberg, Germany. Protein-G Sepharose Fast Flow was purchased from Amersham Biosciences, Freiburg, Germany. Acetonitrile was from Rathburn Chemicals, Walkerburn, Scotland. Trifluoroacetic acid was from Pierce, Rockford, IL, USA. Coomassie Brilliant Blue G, dithiothreitol, iodoacetamide, and 4-hydroxy-α-cyanocinnamic acid were from Sigma, St. Louis, MO, USA, μZiptips were from Millipore, Bedford, MA, USA, the modified porcine trypsin was from Promega, Madison, WI, USA, and ultragrade ammonium bicarbonate was from Fluka, Buchs, Switzerland. All other reagents were of analytical grade.

2.2. Apoptosis induction, Jurkat cell lysate preparation and immunoblotting

Jurkat-E6 cells were maintained in RPMI 1640 medium (Invitrogen Corp., Carlsbad, USA) containing 10% heat inactivated fetal calf serum, penicillin and streptomycin (Invitrogen Corp.) at 37°C in a 5% CO_2 atmosphere. Apoptosis of Jurkat-E6 cells was induced by cross-linking of the CD95 receptor using the anti-APO-1-3 antibody along with 10 ng/ml of protein A for 2 or 4 h.

To prepare Jurkat-E6 cell lysates for immunoprecipitation or immunoblotting, cells were pelleted at $800\times g$ for 2 min, culture medium was removed and the cells were resuspended and washed once in PBS. The cell pellet was then resuspended by vigorous pipetting in ice-cold NP-40 lysis buffer (0,5% NP-40, 50 mM Tris pH 7,4, 150 mM NaCl) with 0,1% protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Lysis was continued for 20 min on ice and the lysates were cleared by centrifugation at $15\,000\times g$ for 10 min at 4°C. The total protein con-

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Abbreviations: hnRNP, heterogeneous ribonuclear protein A2/B1; cPGES, cytoplasmic prostaglandin E2 synthase; ALG-2, apoptosis-linked gene 2; COX, cyclooxygenase; MALDI-TOF, matrix assisted laser desorption ionisation time of flight; MS, mass spectrometry; PG, prostaglandin; HSP90, heat shock protein 90

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centrations of the cleared lysates were determined using a modified Lowry assay (Bio-Rad DC Protein Assay) with bovine serum albumin as the standard.

For SDS–PAGE and immunoblotting, samples were diluted with lysis buffer to balance the protein concentration and then SDS sample buffer was added, followed by heating to 90°C for 2 min. SDS–PAGE was carried out using a 4% stacking gel and a 12% separation gel or a pre-cast 4–20% gradient gel (Cambrex, Bio Science Rockland, Inc., Rockland, MA, USA). The proteins were blotted to Hybond-P (Amersham Biosciences, Uppsala, Sweden) membranes using a semi-dry blotting apparatus with a discontinuous buffer system [4] at room temperature (RT). The membranes were blocked in Tris-buffered saline (TBS) containing 5% dry skim milk (TBSS) and 1% Tween 20 for 45 min at RT. Hybond-P membranes were cut in slices and incubated with primary antibody diluted in TBSS for 1 h at RT in separate trays, washed three times for 10 min each in TBS and incubated with the goat secondary horseradish peroxidase-conjugated antibody for 1 h at RT and finally washed as above. Hybond-P slices were then aligned and the immunoreactivity was visualised using enhanced chemiluminescence (ECL, Amersham Biosciences) followed by exposure to either standard X-ray film (AGFA) or to Hyperfilm (Amersham Biosciences).

2.3. Immunoprecipitation

Prior to immunoprecipitation, the clone 22 antibody was pre-adsorbed to protein-G Sepharose using 4 μg of antibody per 40 μl of pre-washed protein-G Sepharose slurry for 1 h at 4°C, and washed three times in 1 ml of lysis buffer. The lysates to be used for immunoprecipitation were pre-adsorbed with protein-G Sepharose at 4°C for 1 h, and then incubated with the antibody - protein-G Sepharose complexes in a total volume of 1 ml over-night at 4°C The beads were finally washed four times in 1 ml of lysis buffer, and eluted in a small volume of SDS sample buffer by heating to 90°C for 2 min. The eluted proteins were collected in the supernatant after centrifugation at 15 000 × *g* for 5 min, and loaded onto an SDS gel. Following Coomassie Brilliant Blue G staining the bands were cut out, and prepared for matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF-MS).

2.4. In-gel trypsin digestion and MS-analysis

Protein bands were in-gel digested with minor modifications of the protocol by Shevchenko et al., [5]. Excised bands were destained by washing in 25 mM ammonium bicarbonate in 50% acetonitrile. After dehydration using 200 μl of acetonitrile, the samples were reduced with 10 mM DTT in 50 mM ammonium carbonate for 30 min at 56°C. Following dehydration as above the proteins were alkylated using 200 μl of 55 mM iodoacetamide in 50 mM ammonium carbonate for 30 min. Then the samples were dehydrated in 200 μl of acetonitrile and dried in a Speed Vac for 10 min. Samples were rehydrated by addition of 15 μl of 50 mM ammonium bicarbonate with 10 ng/μl of porcine modified trypsin for 15 min, then 10 μl of 50 mM ammonium carbonate were added, and the samples incubated at 37°C over night. Samples were acidified by adding 5 μl of 10% TFA and incubated 10 min. Extracted peptides were removed and 100 μl of 60% acetonitrile were added to the gel slices. After 30 min incubation the supernatants were combined and the acetonitrile removed by lyophilisation. Samples of 10 μl were purified using μ-ZipTips, and the peptides were eluted in 50% of a 1 g/l of α-cyano-4-hydroxycinnamic acid in 0,1% TFA and 50% acetonitrile. 1.5 μl was spotted directly to a Bruker Anchorchip target (400 μm) and analysed on a Bruker UltraFlex (Bruker Daltonics Inc., Billerica, USA). External calibration was done using peptide standards from Bruker Daltonics Inc. MS/MS spectra were acquired on selected peptides using the MS/MS capabilities of the Ultraflex instrument. The peptide mass fingerprint and the MS/MS spectra were searched against the non-redundant MSDB database using Mascot (MatrixScience, London, United Kingdom).

3. Results

3.1. Immunoprecipitation of p23 using the clone 22 anti ALG-2 antibody

Recently, we reported that the clone 22 antibody did not recognise ALG-2, but a protein that appeared 1–2 kDa larger than endogenous human ALG-2 and recombinant murine

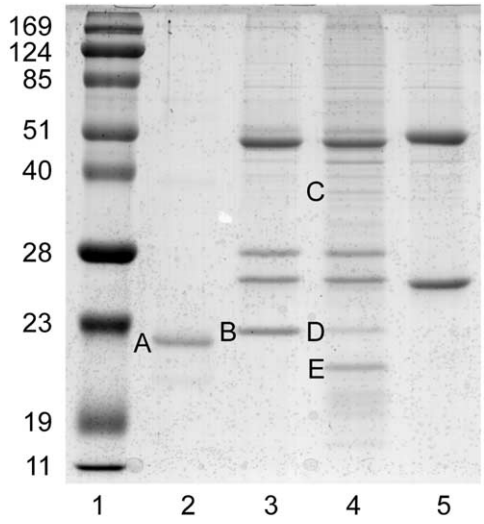

Fig. 1. Specific immunoprecipitation of the co-chaperone p23 protein using the BD clone 22 anti-ALG-2 antibody from non-apoptotic and apoptotic Jurkat-E6 cell lysates. Apoptosis was induced for 4 h using the APO-1-3 antibody. The lanes of this Coomassie stained SDS gel represent: protein molecular weight marker (1), recombinant murine ALG-2 (2), immunoprecipitation from non-apoptotic lysate (3), immunoprecipitation from apoptotic lysate (4), immunoprecipitation using the clone 13 anti-CD95 antibody from non-apoptotic lysate (5) The gel is representative of three separate experiments.

ALG-2, as estimated following SDS–PAGE and immunoblotting [1]. In order to identify the antigen recognised by the clone 22 antibody we tested whether this IgG_1 antibody was suitable for immunoprecipitation from lysates of exponentially growing Jurkat-E6 cells and from lysates of Jurkat-E6 cells induced to undergo apoptosis by CD95 receptor cross-linking. The monoclonal antibody did specifically precipitate proteins, which were visualised by Coomassie staining of an SDS–polyacrylamide gel (Fig. 1, lanes 3 and 4). We observed that the signal intensity of a 23 kDa protein precipitated from the apoptotic Jurkat-E6 cell lysate was decreased (Fig. 1, band D) as compared to that from the non-apoptotic cells (Fig. 1, band B). Moreover, two additional bands of approximately 22 kDa (Fig. 1, band E) and 40 kDa (Fig. 1, band C), respectively, were precipitated from the apoptotic cell lysates. Immunoprecipitation with a p23 unrelated IgG_1 antibody (anti-Fas/CD95/APO-1, clone 13) from the non-apoptotic Jurkat-E6 lysate did not reveal any bands below 25 kDa (Fig. 1, lane 5).

In order to identify the immunoprecipitated proteins the bands designated A–E were cut from the gel and analysed by tryptic in-gel digestion followed by MALDI-TOF-MS and MS/MS analysis of the peptides (Fig. 2). The internal control (Fig. 1, band A) was correctly assigned as murine ALG-2. Band C (Fig. 1) was identified as the human heterogeneous ribonuclear protein A2/B1 (hnRNPA2/B1), and bands B, D, and E (Fig. 1) were all identified as the human progesterone receptor-related protein p23, also named human telomerase binding protein p23, or HSP90 co-chaperone p23 (Fig. 2). Peptides from bands B and E (Fig. 1) were further verified by MS/MS sequencing and were confirmed to derive from p23 (results are summarised in Table 1). These data provide evidence that the clone 22 antibody specifically precipitates p23 but not ALG-2 from non-apoptotic and apopto-

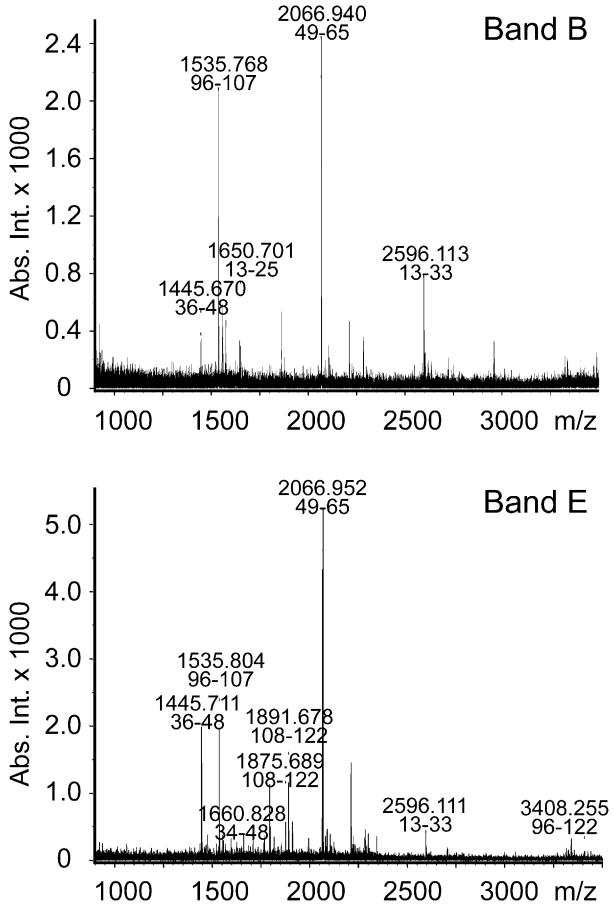

Fig. 2. MALDI-TOF analysis of peptides from bands B and E (indicated in Fig. 1). The immunoprecipitated proteins were separated using SDS–PAGE and the Coomassie stained proteins were cut out of the gel and in-gel trypsin digested. The labelled peaks identify peptides of human HSP90 co-chaperone p23.

tic Jurkat-E6 cells. Therefore, the previously published data on ALG-2 using the clone 22 antibody [6–11] have to be corrected since the investigated protein was indeed the HSP90 co-chaperone protein p23. Furthermore, our data indicate that p23 is cleaved following apoptosis induction by CD95 receptor cross-linking in Jurkat-E6 cells releasing a product of about 22 kDa.

3.2. hnRNPA2/B1 co-immunoprecipitates with p23

The MS-analysis revealed that the clone 22 antibody co-immunoprecipitated hnRNPA2/B1 from the apoptotic Jurkat-E6 cells (Fig. 1, band C). This indicates that p23 is in a complex with the RNA binding protein hnRNPA2/B1. Since immunoprecipitation cannot be considered a quantitative method this does not provide evidence that the p23–hnRNPA2/B1 interaction is specific for apoptosis. hnRNPA2/B1 is involved in splicing and transport of mRNA (see [12]) and it is able to bind to telomeric repeats [13]. A model has been proposed, in which hnRNPs in a complex with the telomerase holoenzyme, HSP90/p23 and other co-factors, is involved in telomere and telomerase regulation [14]. The co-immunoprecipitation of hnRNPA2/B1 and p23, therefore, supports the existence of such a complex.

3.3. p23 is cleaved following apoptosis induction in Jurkat-E6 cells

Treatment of Jurkat-E6 cells with an agonistic anti-CD95 antibody [15] and 10 ng/ml of protein A for 2 h induced severe membrane blebbing and nuclear fragmentation (data not shown), which are hallmarks of apoptosis. To analyse whether the clone 22 antibody would also detect the cleaved p23 in immunoblots and not ALG-2, we compared this antibody with other p23 antibodies and with our two polyclonal anti-ALG-2 antibodies. Fig. 3A shows that the BD clone 22 antibody, the BD clone 16 mouse monoclonal p23 antibody and the p23 clone JJ3 antibody [16] all detect full-length p23 from non-apoptotic Jurkat-E6 lysates and they recognise the full-length p23 and the truncated p23 from the apoptotic Jurkat-E6 lysates. The clone 16 antibody also detects a band around 40 kDa with unknown identity (Fig. 3A). The two anti-ALG-2 antibodies only recognised a single band at 22 kDa in both non-apoptotic and apoptotic Jurkat-E6 lysates (Fig. 3B), which strongly suggests that ALG-2 is not proteolytically degraded following apoptosis induction by CD95 cross-linking. The data give further support for the specificity of the clone 22 antibody, and also show that truncated p23 can be detected by immunoblotting with other p23 antibodies.

4. Discussion

In this paper we show that the BD clone 22 antibody does not recognise ALG-2, but specifically recognises the HSP90 co-chaperone p23. Unfortunately, the clone 22 antibody has been used by several groups to describe properties of ALG-2. These data have to be revised in the light of our new data. However, the work previously done with the clone 22 antibody significantly contributes to the understanding of novel functions of p23.

Table 1
Identification of peptides after in-gel tryptic digestion of bands A–E (see Fig. 1) by MALDI-TOF-MS

Band	Accession number	MS coverage	MS/MS residues	Protein name	Gene
A	P12815	85.9%	–	Mouse programmed cell death protein 6/apoptosis linked gene 2 (ALG-2)	PDCD6
B	Q15185	40.6%	36–48 96–107 49–65 13–33	Human progesterone receptor-related protein p23/HSP90 co-chaperone protein p23	TEBP
C	P22626	49.6%	–	Human heterogeneous nuclear ribonucleoprotein A2/B1	ROA2
D	Q15185	50.0%	36–48	Human progesterone receptor-related protein p23/HSP90 co-chaperone protein p23	TEBP
E	Q15185	41.9%	–	Human progesterone receptor-related protein p23/HSP90 co-chaperone protein p23	TEBP

The MS coverage indicates the percentage of the protein that was identified by MS analysis alone. Peptide residues identified using MS/MS are also listed.

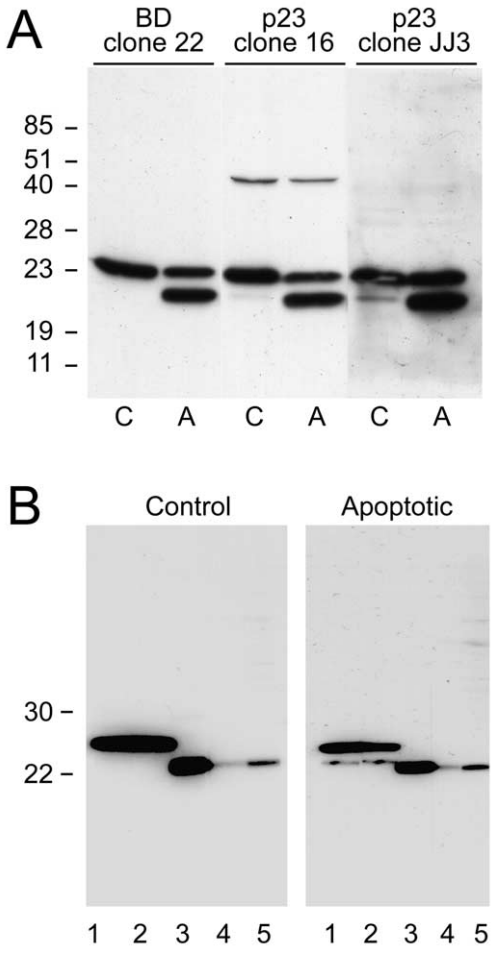

Fig. 3. The co-chaperone p23 is cleaved following CD95 cross-linking in Jurkat-E6 cells. A: Immunoblot of control (non-apoptotic) and apoptotic Jurkat-E6 cell lysates prepared after 4 h of apoptosis induction. The BD anti-ALG-2 clone 22, the BD anti-p23 clone 16, and the anti-p23 clone JJ3 antibodies were diluted 5000, 1000, and 1000 times, respectively. The immunoblot is representative of two separate experiments. B: Immunoblot of control (non-apoptotic) and apoptotic Jurkat-E6 cell lysates prepared after 2 h of apoptosis induction. The BD clone 22 antibody in lanes 1 and 2, is diluted 3000 and 5000 times, respectively, The affinity purified rabbit anti-recombinant ALG-2 antibody in lane 3, and the affinity purified rabbit anti-peptide antibody in lanes 4 and 5, are diluted 1000, 500, and 200 times, respectively. The immunoblot is representative of four separate experiments.

4.1. Increased expression of p23 after rat focal cerebral ischemia

In an animal model of temporary focal ischemia, occlusion of the rat right carotid artery for 20 or 90 min was used to induce focal ischemia and this was followed by a reperfusion period for up to 72 h [8]. Increased expression of p23 was observed in the frontal cortex and in the parietal cortex within 24 h of reperfusion by both immunohistochemistry and immunoblotting using the clone 22 antibody. Double staining revealed that p23 over-expression co-localised with DNA fragmentation. Interestingly, both COX isoforms (COX-1 and COX-2) have been shown to be involved in the progression of neuronal damage following ischemia [17]. The observed p23 up-regulation might contribute to increased PGE_2 synthesis, which is known to elevate the levels of free radicals leading to oxidative damage. It remains to be investigated whether up-regulation of p23 in ischemia leads to elevated PGE_2 concentrations and whether p23 might be the rate-limiting factor.

4.2. p23 co-immunoprecipitates with CD95 and is cleaved in apoptosis

Following apoptosis induction by CD95 crosslinking in Jurkat-E6 cells, p23 was co-precipitated with CD95 and was shown to be cleaved using the clone 22 antibody [9]. p23 cleavage coincided with poly-ADP-ribose polymerase cleavage assumed to be mediated by executioner caspases during apoptotic cell death. This could indicate that p23 is a target for these caspases. Using the clone 22 antibody in immunoprecipitation and immunoblotting experiments, we have confirmed that apoptosis induction by CD95 cross-linking in Jurkat-E6 cells leads to the cleavage of p23 (Fig. 1 and 3). These observations point to a new link between apoptosis and the demise of HSP90 chaperone client proteins which are dependent on the HSP90 p23 co-chaperone complex to maintain their proper function. HSP90 chaperone activity is needed for the correct folding and stability of several different signalling proteins including multiple kinases and steroid receptors (see [18]). Interestingly, it has also been demonstrated that the PGE_2 synthase activity of cPGES is regulated by HSP90 providing a link between the synthesis of PGE_2 and a molecular chaperone [19]. The chaperone functions of p23 are still not known in details, but both passive chaperone activity of p23 itself [20] and HSP90 co-chaperone activity have been described. The removal of p23 by proteolysis during apoptosis may be part of the cooperative actions of the caspases that leads to the well-defined apoptotic phenotype.

4.3. Down-regulation of p23 in atherosclerotic plaques

A comparison between atherosclerotic human carotid endarterectomised specimens and non-atherosclerotic arteries using a commercial Western blot array showed that the down-regulation of the clone 22 antigen in the atherosclerotic plaques was one of the most prominent differences in protein expression [11,28].

At the moment little is known about the biochemical processes involved in atherosclerosis leading to myocardial infarction and stroke. Inflammation in the arterial wall plays a crucial role in pathogenesis of myocardial infarction caused by rupture of an atherosclerotic plaque. The anti-inflammatory and anti-thrombotic drug aspirin, that inhibits COX 1 and prevents thromboxane A2 synthesis at the platelet-vessel wall, is known to decrease the incidence of cardiovascular disease (see [21]). The PGE_2 production downstream of COX-1 that is mediated by p23 could have an important role. Severe down-regulation of p23 could lead to an disturbed balance between the PGH_2 metabolising enzymes and a changed prostanoid status, eventually towards a pathogenic situation. Further investigation of the role of p23 should be conducted in order to understand the role of p23 in atherosclerosis, especially in the light of the central enzymatic role of p23 as one of the two known PGE_2 synthases. Since massive apoptosis in the atherosclerotic plaques has been described (see [22]), it is likely that the low level of p23 is the result of caspase activity in the plaques. However, down-regulation of p23 could also be a trigger of apoptosis.

4.4. PYK2

In a study of the interaction between ALG-2 binding pro-

tein 1 (AIP1) and a protein expressed in tumourigenic astrocytes (SETA) [10], it was found that the focal adhesion kinase 2 (FAK-2 or PYK2) can be co-immunoprecipitated with the clone 22 antigen. Since p23 is a HSP90 co-chaperone, PYK2 should be added to the growing list of HSP90 chaperone complex client proteins, which also includes several important signalling kinases.

4.5. Over-expression of p23 in cancer tissue

By comparison of normal tissue and adjacent malignant tissue of mammary carcinomas as well as metastases, it was reported using the clone 22 antibody that the antigen expression was low or absent in normal tissue, increased in the primary tumour and strongly up-regulated in the metastases [7].

Recent studies have shown that COX-1 is up-regulated in several human cancers, including breast [23], prostate [24] and ovarian cancer [25], as well as in cervical carcinomas [26]. In a mouse model of a highly metastatic mammary carcinoma, the oral intake of either selective COX-1 inhibitors or COX-2 inhibitors resulted in significant inhibition of tumour growth [27]. Elevated synthesis of PGE_2 has been shown to induce a malignant phenotype with an increased metastatic potential of epithelial cells, to inhibit apoptosis, and to promote angiogenesis (see[26]). Therefore, the observed up-regulation of p23 in malignant tissue, suggests that the inhibition at the level of the COXs, could be improved by more specific targeting of p23.

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