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Transient activation of the c-Jun N-terminal kinase (JNK) activity by ligation of the tetraspan CD53 antigen in different cell types

Mónica Yunta[1], José L. Oliva[2], Ramiro Barcia[1], Vaclav Horejsi[3], Paula Angelisova[3] and Pedro A. Lazo[1]

[1]*Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, C.S.I.C. Universidad de Salamanca, Spain;* [2]*Unidad de Biología Celular, Centro Nacional de Biología Fundamental, Instituto de Salud Carlos III, Majadahonda, Spain;* [3]*Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic*

The CD53 antigen is a member of the tetraspanin membrane protein family that is expressed in the lymphoid-myeloid lineage. We have studied the implication of CD53 antigen in signal transduction by determining the effect of its ligation on the c-Jun N-terminal kinase (JNK) in different cell types. Ligation of the rat or human CD53 antigen induces a three- to fourfold transient activation of JNK activity that peaks at 3–5 min. The effect was detected by assaying the endogenous or exogenous (transfected) JNK activity. The JNK response was detected in IR938F cells, a rat B-cell lymphoma, and in Jurkat cells derived from a human T-cell lymphoma. This JNK activation was not mediated by the *vav* oncogene, and CD53 does not cooperate with CD3 for *vav* activation. A similar JNK activation was also detected in a human renal carcinoma cell line that was transiently transfected with the human CD53 cDNA to mimic the CD53 ectopic expression in carcinomas. In stable CD53-transfected cells it stimulated Jun-dependent transcriptional activity. We conclude that parts of the cell responses modulated by the CD53 are mediated by JNK activation, and this activation is independent of the different protein interactions that the CD53 protein has on specific cell types.

Keywords: CD53; JNK; Jun kinase; tetraspan antigen; signal transduction.

Tetraspanin proteins are a group of integral membrane proteins, with four transmembrane domains, that were defined by their structural characteristics. Among these proteins are CD9, CD37, CD53, CD63, CD81, CD82, CD151, NAG2, uroplakin, and SAS [1]. These proteins are expressed in different cell types, such as lymphoid, epithelial and muscle cells, but do not have any clearly defined biological function [1,2]. Tetraspanin proteins can influence several biological processes, such as cell motility [3,4], and homotypic adhesion [5–11]. However, the mechanisms by which these antigens contribute to the modulation of these processes are not known. These roles might be partly accounted for by the interactions between tetraspanin proteins and other membrane proteins. Tetraspanin antigens located on the cellular membrane have been detected both as free molecules, or interacting with either other tetraspanin proteins, or integrins, particularly those with the β1 subunit [12–15], MHC class II antigens [16–18], T-cell receptor [19], CD19 molecules [14] and members of the immunoglobulin super family [20,21]. In these protein–protein interactions, the tetraspanin antigens have been proposed to play a costimulatory role [10,22]. Because of these protein interactions, tetraspanin antigens can influence intracellular signalling pathways. The ligation of CD53 has been shown to induce intracellular calcium mobilization in different cell types, such as human B cells and monocytes [23,24] and rat macrophages [6,25].

The surface of normal cells displays a complex pattern of tetraspanin antigens, with at least six different proteins present in a specific cell type, suggesting that they form a tetraspanin complex composed of different subunits, as detected in Burkitt lymphoma cells [26], and other cell types from which they can be coimmunoprecipitated [12,27]. However, in tumour biology these antigens have been studied individually. Reduction in antigen levels have been correlated with poor tumour prognosis [28], such as is the case for CD9 in lung carcinoma [29], CD82 in prostate carcinoma [30], or CD63 in melanoma [28]. The role of CD9 has been related to its modulation of cell motility, as the reintroduction of CD9 in the cell functions as a brake [31]. In addition, CD53 deficiency has a clinical phenotype similar to those of inherited defects of cell adhesion molecules [32]. Because of the complex tetraspanin pattern of gene expression, it is very likely that the adhesion and migration properties of tumours are conditioned by the alteration in the composition of cell-specific expression patterns [33,34]. CD53 antigen expression is restricted mainly to the lymphoid-myeloid lineage, with very low levels in other cell types [1,2]. CD53 is proteolytically down-regulated when human neutrophils are stimulated with different chemotactic stimuli, such as platelet activating factor or flip [35]. However, CD53 is expressed at very high levels in some carcinomas, such as pancreatic cancer (M. Yunta & P. Angelisova unpublished data). The CD53 ectopic expression might facilitate tumour migration by the lymphatic system, or reflect a phenotype of resistance to radiation, as has been demonstrated by the over-expression of CD53 [36].

Correspondence to P. A. Lazo, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain. Fax: + 34 923 294795, Tel.: + 34 923 294804, E-mail: plazozbi@usal.es

Abbreviations: JNK, c-Jun N-terminal kinase; HA, haemagglutinin; GST, glutathione *S*-transferase; PKC, protein kinase C.

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Our knowledge of the signalling role of tetraspan antigens is rather limited. However, both protein kinase C (PKC) [6,25,37,38] and phosphatidylinositol 4-kinase [39] appear to be implicated. Because the CD53 signal implicates *de novo* transcription [6,25], we have studied the possibility that activation of the N-terminal Jun kinase (JNK) activity might be a component of the cellular response to CD53 antigen ligation, as suggested by the induction of genes regulated by Jun [40], such as the inducible form of nitric oxide synthase, also induced by CD53 in rat macrophages [25]. JNK has been studied mostly in the context of cellular responses to stress, but it is also implicated in proliferation, differentiation, and cell death [41–44]. In lymphoid cells part of these signals are mediated by the *vav* oncogene [45]. The phosphorylation of Jun in its N-terminus has been shown to protect cells from going into apoptosis [46]. We show that ligation of the CD53 antigen by itself is able to induce a fast and transient activation of the JNK activity that is not mediated by the *vav* oncogene, and does not cooperate with CD3 in Vav phosphorylation. This activity could also be induced in situations where the CD53 antigen is expressed ectopically, as occurs in some tumour cells where it might be an indicator of resistance to treatment. This JNK activation induces c-*jun* dependent transcription. The CD53 effect on JNK activity appears to be independent of the cell type, and thus of cell-specific protein interactions.

MATERIALS AND METHODS

Cell lines

The rat IR938F, a pre-B cell lymphoma, and the human Jurkat cell line, derived from an acute T-cell lymphoma, were grown in RPMI1640 supplemented with 10% foetal calf serum. The human renal carcinoma 293T cell line was grown in DMEM media supplemented with 10% foetal calf serum. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

Plasmids

The human CD53 full-length cDNA was subcloned as a *Bam*HI–*Bgl*II fragment in vector pCEFLZ-KZ (S. Gutkind, NIH, Bethseda, MD, USA) under the control of the cytomegalovirus promoter. The clone was named pCEFL-KZ-CD53. For experiments using exogenous Jun kinase, a clone with the N-terminus of Jun kinase containing the haemagglutinin (HA) epitope tag (clone pHA-JNK from S. Gutkind, NIH, Bethseda, MD, USA) under the control of the cytomegalovirus promoter was used for transfections. The HA epitope was used for immunoprecipitation, detection in Western blots, and quantification. For reporter assays of luciferase activity the following plasmids were used, the 5 × Gal4-Luc (reporter), and Gal4-c-Jun (1–223) and Gal-c-Jun (1–223, S63/73A) expression vectors encode fusion proteins containing the GAL4 DNA-binding domain and the c-Jun activation domain (residues 1–223) in wild-type and mutant forms (not phosphorylatable by mutation of both serines 63 and 73 to alanine). These plasmids were kindly provided by M. Karin (University of California, San Diego, CA, USA). As an internal control for transfection efficiency and normalization we used the Renilla reporter plasmid pRL-tk. The generated light was detected with an OPTOCOM-1 luminometer (MGM Instruments, Inc., Hamden, CT, USA).

Transfections

For the transfection of Jurkat cells, a human T-cell lymphoma cell line, the cells were grown to a density of 5×10^5 cells·mL^{-1}. For each time point 3×10^6 cells were used. The cells were washed in OPTIMEM (Life Technologies) and resuspended in 800 μL of OPTIMEM. The transfection mix was prepared with 100 μL OPTIMEM with 15 μL lipofectamine (Life Technologies) and 100 μL of OPTIMEM with 10 μg of plasmid DNA. The two were mixed for 45 min, added to the cells and put in the incubator for 5 h. Cells were then washed in NaCl/Pi and resuspended in normal culture medium with 10% FBS. In that way more than 60% of the cells were viable, and 15–20% were transfected as determined by flow cytometry. Forty-eight hours after transfection, the cells were starved for 2 h in culture medium with 0.5% FBS to reduce the background of endogenous kinase activity. The starved cells were stimulated by ligation with mAb as indicated in the experiments. Cell lysis was carried out in 25 mM Hepes pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton-100, 20 mM β-glycerophosphate, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM dithiothreitol, 0.1 mM sodium vanadate, 2 μg·mL^{-1} leupeptin, 2 μg·mL^{-1} aprotinin, and 100 μg·mL^{-1} phenylmethanesulfonyl fluoride. After incubating for 15 min on ice, the cells were centrifuged to pellet the debris, and the supernatant was used for immunoprecipitation and kinase assays. The human renal carcinoma 293T cells were also transfected using OPTI-MEM and Lipofectamine, cells were lysed on the dishes and immunoprecipitated as indicated.

Antibodies

Four mAbs against human CD53 were used, MEM53 (IgG1) isotype) [47], and 202–24b, 161–2 and 63–5A3 (IgG1, IgG2a and IgG2b isotypes, respectively) from R. Vilella (University Hospital Clinic, Barcelona, Spain). To detect the rat CD53 antigen the MRC OX-44 mAb was used (Serotec). To detect the HA epitope used for tagging, and present in transfected JNK molecules, we used the HA.11 antibody from BABCO (Richmond, CA, USA). Against CD3 we used the clone UCHT1 antibody (DAKO). The anti-Vav antibody was kindly provided by X. Bustelo (SUNY, Stony Brook, NY, USA). To detect phosphorylation of Vav we used the PY99 antiphosphotyrosine antibody (Santa Cruz, CA, USA). The cell phenotype was determined by flow cytometry with a FACScalibur cytometer (Becton-Dickinson).

Immunoprecipitation and Western blots

For efficiency of transfection and quantification, cells were immunoprecipated with an antibody against the marker epitope, to determine the level of transfected protein. For this, the cleared cellular lysate was mixed with the anti-HA antibody and Gammabind-Plus-sepharose (Amersham Biosciences) for 1 h at 4 °C with rotation. The pellet was washed first with NaCl/P_i containing 1% NP40 and 2 mM Na orthovanadate. Next it was washed in 100 mM Tris HCl

pH 7.5, 0.5 mM LiCl, and three times in kinase reaction buffer (12.5 mM Mops pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na orthovanadate). The products were analysed by SDS/PAGE under denaturing conditions and transferred to Immobilon-P membranes (Millipore). The membranes were blocked with 5% skimmed milk in $NaCl/P_i$, and then incubated with the specific antibody, followed by a rabbit antimouse IgG with peroxidase and developed with an ECL chemiluminescence kit (Amersham). The films were digitized at high resolution in a UMAX scanner.

In vitro JNK assays

Kinase assays were performed in kinase reaction buffer with 10 μCi [γ-^{32}P]ATP, 20 μM ATP, 3.3 mM dithiothreitol and 4 μg specific substrate fusion protein, either glutathione *S*-transferase (GST)–Jun (from M. Karin, University of California, San Diego, CA, USA) or GST-ATF2 (from S. Gutkind, NIH, Bethesda, MD, USA). The kinase reaction was carried out at 37 °C for 30 min The phosphorylated products were analysed by SDS/PAGE and the radioactivity was quantified directly using a FUJIBAS phosphorimager system (Fuji). JNK phosphorylation was induced in controls with 10 $\mu g \cdot mL^{-1}$ of anisomycin or cycloheximide for IR938F and Jurkat cells in suspension. For adherent 293T cells JNK was induced by UV light. All positive controls were used for establishing that JNK was functional in the system, but the way they induce JNK activation is different from that of tetraspanin antigens. The activation of JNK was normalized with respect to the efficiency of transfection, as determined by the use of specific antibodies and their detection by luminescence with an ECL kit (Amersham-Pharmacia). The relative increases in activity were always referred to the unstimulated cells. All experiments were performed at least four times unless indicated otherwise; the mean and their standard deviation and their statistical significance by Student's *t*-test were determined. In cells that grow as a monolayer the positive control was induced by treatment of the cells with a 25 $J \cdot m^{-2}$ dose of UV light by irradiation with a Stratalinker (Stratagene).

Luciferase assays of transcriptional activation

Cell extracts to measure the reporter luciferase activity and the internal Renilla activity were determined using the Dual Luciferase Reporter Assay system from Promega as described previously [48].

RESULTS

MRC OX-44 induces activation of JNK in rat IR938F cells

The rat IR938F cell line is derived from a pre-B-cell lymphoma that expresses high levels of the OX-44 (rat CD53) antigen [37]. This cell line has been shown previously to respond to OX-44 antigen ligation with the mAb MRC OX-44 [6,37]. The signal generated appeared to implicate PKC in the IR938F cell line [37], and in normal rat macrophages also there was generation of diacylglycerol and inositol-1,4,5-trisphosphate [25]. Among the biological effects observed are the induction of homotypic adhesion, which was mediated by both PKC-dependent and -independent pathways [6]. This effect of homotypic adhesion can also be induced by ligation of other tetraspanin antigens, such as CD9, CD81 and CD82, with their corresponding antibodies [10], and thus these proteins may mediate a common effect.

Therefore, we first tested if antibody ligation of the OX-44 antigen was able to elicit an intracellular signal that might implicate the JNK activity. IR938F cells were stimulated at different times with 10 $\mu g \cdot mL^{-1}$ of mAb MRC OX-44, a concentration similar to that required for rapid induction of other biological effects [6,25,37]. The endogenous JNK activity was determined in whole cell extracts using as specific substrate the GST–Jun fusion protein [49]. The ligation of rat CD53 antigen with MRC OX-44 mAb induced a transient activation of JNK, as shown by the incorporation of radioactivity in the fusion protein, which reaches a significant threefold increase at 3–5 min after antibody addition (Fig. 1).

Human CD53 antigen ligation activates endogenous and exogenous JNK activity in Jurkat cells

To determine if the activation of JNK by ligation of the human CD53 antigen was a common signal response shared with other cells of the lymphoid lineage, we used the Jurkat

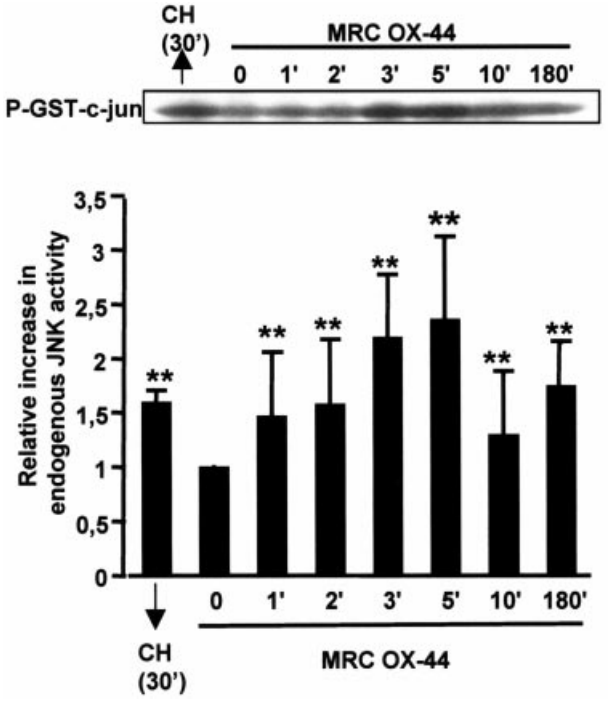

Fig. 1. Activation of the endogenous JNK activity in rat IR938F immunocytoma cells. The cells were stimulated with 10 μg mAb MRC OX-44 (antirat CD53) for the indicated times. The GST–Jun fusion protein was used as substrate in the assay of endogenous JNK present in whole cell extracts. At the top is the autoradiography of the phosphorylated GST–Jun protein in an individual experiment to illustrate the increase in activity following CD53 ligation and detected between 3 and 5 min. At the bottom is shown the quantification of relative increase in endogenous JNK activity with respect to nonstimulated cells (0′). The mean values with their SD of the four independent experiments are shown; $P < 0.001$ (**). The positive control for activation used in these experiments was cycloheximide (CH).

cell line, derived from a human acute T-cell lymphoma, that expresses high levels of the CD53 antigen (determined by flow cytometry). First, we analysed if the endogenous JNK activity was able to respond to ligation of the antigen with the MEM53 (anti-human CD53) mAb. The response was also a threefold activation of the endogenous JNK, detected with GST–Jun as substrate, which reached a maximum at 2–3 min after antigen ligation (Fig. 2A). However, Jun phosphorylation could also be mediated by the p38 kinase when using endogenous kinase activity. To overcome this possibility and to confirm the role of JNK, we transfected Jurkat cells with exogenous JNK protein tagged with the HA epitope: in that way we could separate its activation from other endogenous kinases. After stimulating the transfected cells by CD53 ligation, the HA-tagged JNK kinase was immunoprecipitated from whole cell extracts with an anti-HA antibody to separate it from the endogenous kinase. After separation, the kinase activity was determined in the immunoprecipitate using two different substrates, GST–Jun and GST–ATF-2 fusion proteins (Fig. 2B), two of its well characterized physiological targets. The activity was normalized with respect to the amount of HA-JNK transfected protein present in the immunoprecipitate, which was determined with the mAb against the HA epitope tag. The activation of the exogenous or transfected JNK was similar, in both time and magnitude of the response, to that of the endogenous kinase. Therefore, we concluded that antibody ligation of the human CD53 antigen can also induce a similar activation of JNK in Jurkat cells, a different cell type.

JNK activation in Jurkat cells is not mediated by Vav

The *vav* oncogene is a major transducing molecule in lymphocyte signalling [45,50], and in some cells JNK activation is mediated by Vav signalling [51]. Therefore, we tested if Vav phosphorylation is a mediator of the signal generated by CD53 antigen ligation. In these experiments we used Jurkat cells, in which JNK activation is known to be induced by ligation with anti-CD3 antibodies, and this activation is enhanced very strongly by coligation with antibodies against CD28 [52,53]. The specific phosphorylation of Vav was determined by immunoprecipitation with an anti-Vav antibody, followed by a Western blot with an anti-phosphotyrosine antibody (PY99). We first determined that MEM53 by itself was not able to induce phosphorylation of the Vav protein, but it was phosphorylated in the positive control with an anti-CD3 antibody (Fig. 3A). Next we performed a titration of the Vav phosphorylation as a CD3 response in these cells, to select an antibody concentration

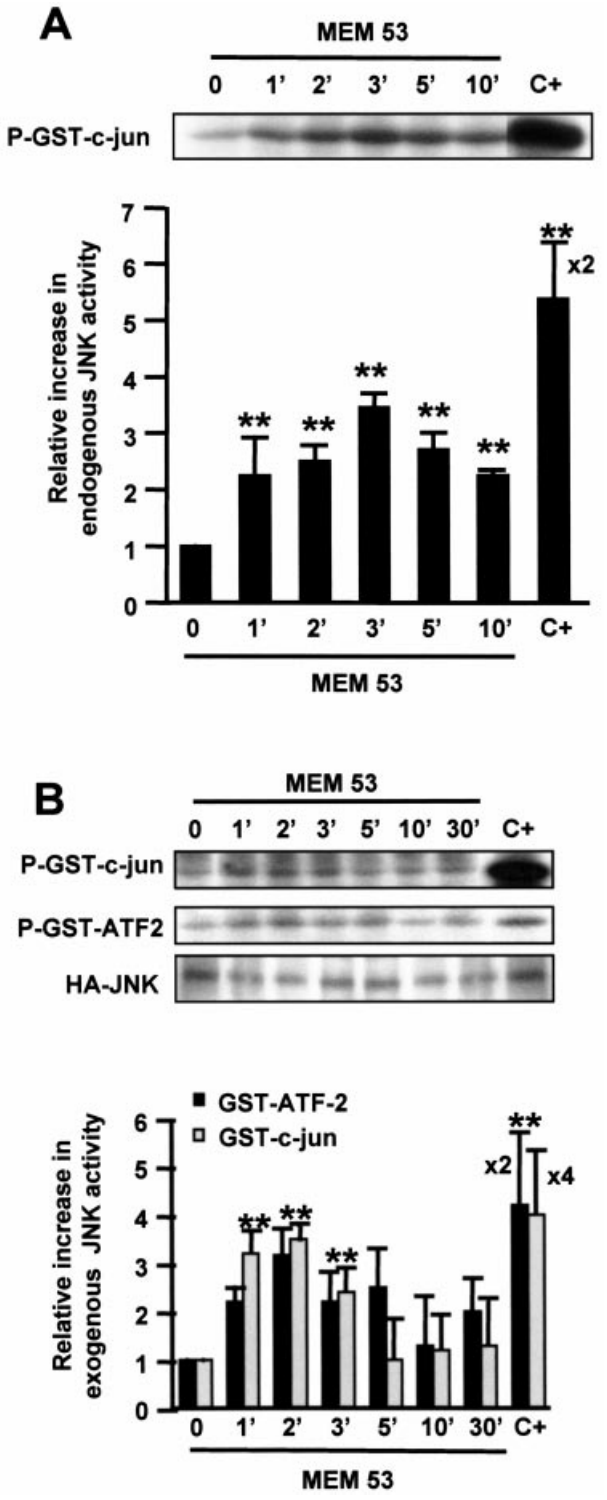

Fig. 2. Activation of endogenous JNK (A) and exogenous or transfected HA-JNK (B) by CD53 ligation in Jurkat cells. Jurkat cells were stimulated with 10 μg MEM53 mAb (anti-human CD53). The endogenous activity was determined by adding an excess of GST–Jun substrate to whole cell extracts. The exogenous activity (transfected JNK with the HA epitope) was determined with GST–Jun and GST–ATF2 as substrates and the activity was determined in the anti-HA immunoprecipitate. The controls for transfection and immunoprecipitation was determined by a Western blot using the antibody against the HA epitope. The blots represent individual experiments. The diagrams with bars represent the means of four independent experiments with the SD; $P < 0.001$ (**). In (A) the relative increase was determined with respect to the nonstimulated cells (point 0′). In (B) the quantification was determined by the ratio of the signal of the radioactivity in the GST–Jun and GST–ATF2 fusion proteins with respect to the signal for the HA epitope. As reference for the increases, the value in nonstimulated cells was used as one. As positive control for the inducibility of the activation we used anisomycin (lane C+); $P < 0.001$ (**). The mean values of the positive controls should to be multiplied by the factor indicated at the side of the bar.

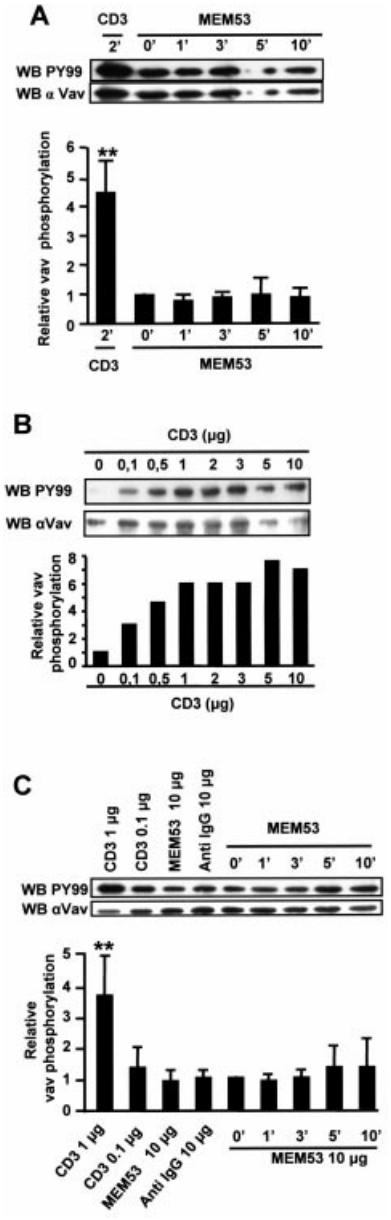

Fig. 3. Effects of CD53 ligation on Vav phosphorylation. (A) Effect of CD53 ligation with 10 μg MEM53 mAb on Vav phosphorylation. Ligation of CD3 was used as the positive control. At the top is a gel from one experiment, showing incorporation of phosphate detected with the PY99 mAb and the total amount of Vav protein detected by Western blot. The ratio of the PY99 to the Vav signal in Western blotting was used for quantification. The ratio at time 0′ was used as the reference value for the increases. The bars represent the means of three independent experiments. (B) Effect of different concentrations of anti-CD3 mAb on Vav phosphorylation. Cells were stimulated for 3 min. This experiment for dose selection was performed only once. (C) Cross-linking of anti-CD53 and anti-CD3 mAbs at suboptimal concentrations of anti-CD3. In all cases the extract was precipitated with an anti-Vav polyclonal antibody and developed with an anti-phosphotyrosine (PY99) antibody. All the bands in the gel were quantified after scanning in a phosphorimager system. Values are the mean of four experiments with the SD; $P < 0.001$ (**).

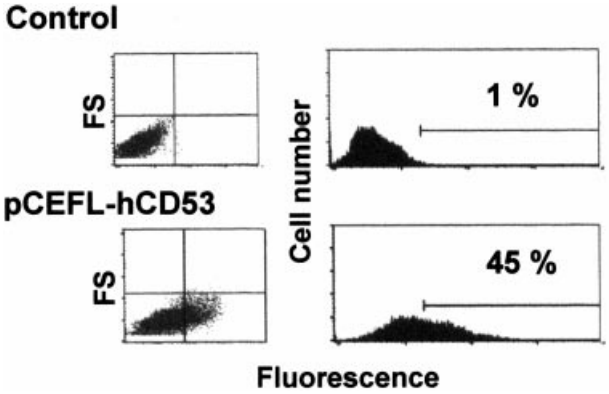

Fig. 4. Ectopic expression of human CD53 antigen in carcinoma 293T cells. In the panels at the top the control is shown, and at the bottom the cells transfected with human CD53 are shown.

that is suboptimal for Vav phosphorylation, and that could be used to study the possibility of costimulatory signals: the selected anti-CD3 concentration was 0.1 $\mu g \cdot mL^{-1}$ (Fig. 3B); this suboptimal concentration of anti-CD3 was the same that required for costimulation of CD3 in the response to CD28 ligation [52]. Finally we studied if, using this suboptimal concentration of anti-CD3 antibody with MEM53 that were cross-linked, the Vav phosphorylation response could be potentiated. The cross-linking of these two antibodies did not costimulate the signal that induces Vav phosphorylation (Fig. 3C). Therefore we concluded that the signal generated by MEM53 is not mediated via the *vav* oncogene, and does not cooperate with CD3 in its activation; therefore the CD53 role, as costimulatory molecule, must be mediated by an independent signalling pathway.

Ligation of ectopic CD53 antigen in 293T cells activated JNK

The human CD53 antigen is ectopically expressed in some carcinomas, and might be related to the migration properties of carcinoma cells by the lymphatic system, and to the generation of lymph node metastasis. Some tetraspanin antigens have been shown to modulate the migration and metastatic properties of tumour cells [34,54]. To mimic this ectopic expression, the full-length human CD53 cDNA under the control of the cytomegalovirus promoter (pCEFL-KZ-hCD53), was transfected into human 293T cells derived from a renal carcinoma. The transfected cells were analysed by flow cytometry for the presence of human CD53 antigen expression. Forty-eight hours after transfection there was a displacement of the fluorescence peak, and 45% of the cells where within the positive window (Fig. 4).

Therefore, as these transiently transfected 293T cells express the CD53 antigen ectopically, we proceeded to determine if ligation of the ectopic CD53 molecule, out of its normal lymphoid context, could also have an effect on JNK activity. For this purpose 293T cells were transiently cotransfected with pCEFL-KZ-CD53 and pHA-JNK plasmids. Forty-eight hours after transfection, the cells were placed in serum-free medium for 2 h to reduce endogenous kinase activity, without compromising cell viability, and afterwards the cells were stimulated with 10 μg of the mAb MEM53. The activation was determined using the GST–

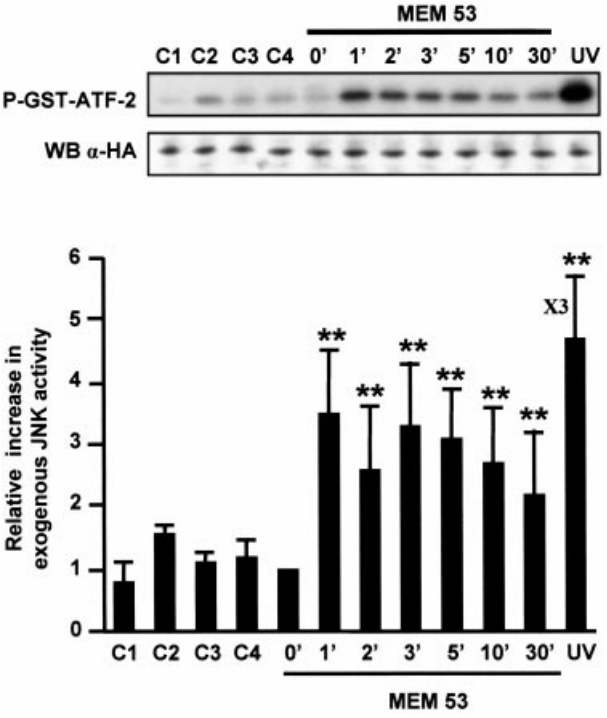

Fig. 5. Activation of exogenous HA-JNK activity by MEM53 ligation of the hCD53 antigen in 293T transfected cells. After stimulation the cells were lysed and immunoprecipitated with an anti-HA epitope antibody. JNK activity was measured in the immunoprecipitate by the incorporation of radioactivity in the GST–ATF2 fusion protein used as substrate. At the top is the assay of the transfected HA-JNK activity detected in the anti-HA immunoprecipitate in an individual experiment. At the bottom is the quantification of the level of the activation of JNK activity induced by CD53 ligation in three independent experiments. The relative values are calculated by the ratio of the incorporation of radioactivity in GST–ATF-2 with respect to the signal of the HA epitope in the immunoprecipitate measured by chemiluminescence. C1, Cells transfected with vector; C2, cells transfected with vector and stimulated with MEM53 for 3 min; C3, cells stimulated for 3 min with IgG1 isotype matched antibody; C4, cells stimulated for 30 min with IgG1 isotype-matched antibody. Time ranged for 0–30 min. As positive control we used irradiation by UV light (25 $J \cdot m^{-2}$) as described in the Methods. Values are the means of four experiments with the SD; $P < 0.001$ (**).

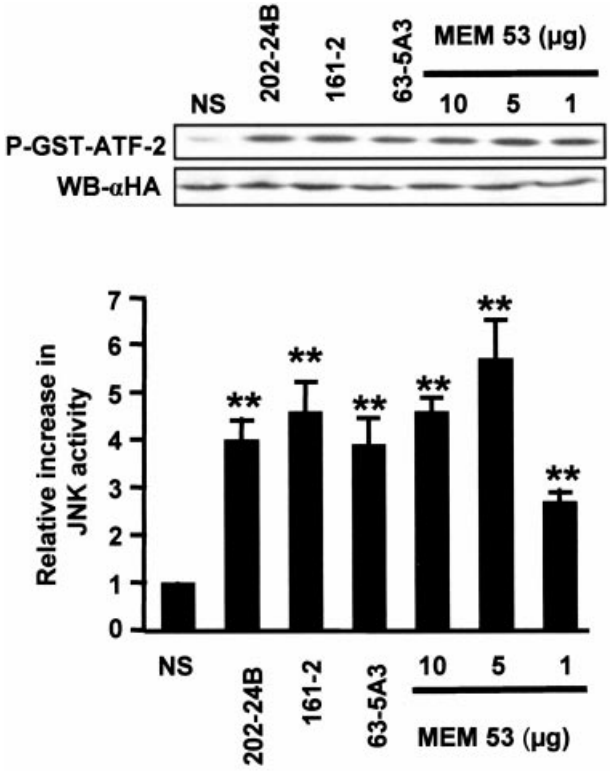

Fig. 6. Ligation of CD53 with different anti-human CD53 mAbs induces a similar effect in CD53-positive 293T cells. Cells were stimulated with 10 μg of 202-24B, 63-5A3, 161-2 mAb and three different concentrations of MEM53 mAb. The gel shows the phosphorylation of the GST–ATF-2 substrate by the kinase in the HA-JNK immunoprecipitate after stimulation by ligation for 3 min. At the top are the blots of an individual experiment, and at the bottom is the quantification of the increase in incorporation of radioactivity detected in the GST–ATF2 fusion protein with respect to the HA epitope. The results are the mean of four independent experiments with the SD; $P < 0.001$ (**). NS: Nonstimulated.

ATF2 fusion protein as substrate of the exogenous HA-JNK activity that was measured in the immunoprecipitate (Fig. 5). The incorporation of radioactivity was normalized to the level of HA-JNK transfected into the cells and determined by developing a Western blot with anti-HA antibody. CD53 antigen ligation induces a fourfold increase in JNK activity, with the peak of activity at 1–3 min; thus it is a fast and transient activation. Such activation was not detectable in cells transfected with the empty vector, and stimulated with the antibody, or in isotype-matched controls (Fig. 5).

Different anti-CD53 antibodies induced a similar effect

To demonstrate further that the stimulation of JNK activity is independent of the specific mAb used in the experiments, 293T cells transfected with the pCEFL-KZ-CD53 plasmid were stimulated with another three different mAbs against the human CD53 antigen, and their effects on JNK activity were compared with those of MEM53 after stimulation for 3 min. As shown in Fig. 6, the three antibodies activated the JNK activity to a level similar to that obtained with the high dose of MEM53 mAb. The activity was determined as the incorporation of ^{32}P in the GST–ATF2 fusion protein substrate, and was normalized with respect to the amount of the HA epitope present in the HA-JNK immunoprecipitate used for the kinase assay. The magnitude of the increase in activity was fourfold in all cases, except when smaller amounts of MEM53 were used (Fig. 6). We concluded that the activation of JNK is a consequence of engaging the CD53 cell surface molecule by a ligand, which in these experiments were different mAbs. Natural ligands of CD53 or other tetraspan proteins are not yet known.

Activation of Jun dependent transcription by CD53

Activation of the Jun transcriptional role depends on its previous phosphorylation by JNK [46]. Because the effect of CD53 antigen ligation on JNK activation appeared to be cell type independent, we determined if this activation does

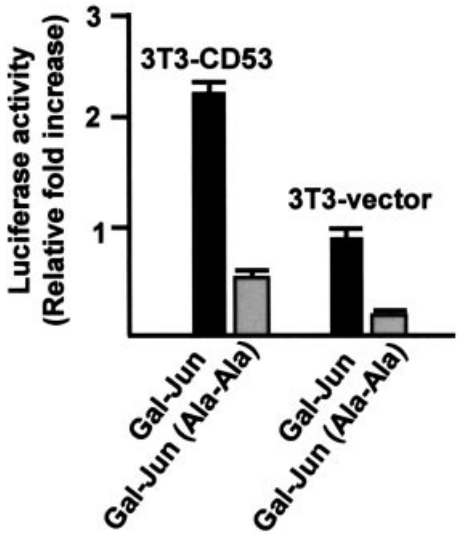

Fig. 7. Stimulation of Jun-dependent transcriptional activity by CD53 antigen ligation. NIH3T3 cells stably expressing the CD53 antigen (left panel) or control cells with the empty vector pMEX-neo (right panel) were transiently transfected with activatable Gal4-c-Jun or its dominant negative mutant Gal4-c-Jun (S63/73A, not phosphorylatable), as well as with the reporter plasmid 5xGAL4-Luc. After serum deprivation to lower endogenous JNK activity, the cells were incubated for 6 h in the presence of MEM53 antibody. The luciferase activity was corrected for the efficiency of transfection by determining the activity of plasmid pRL-tk using a Renilla dual luciferase assay system. The results are the mean of three independent experiments with the SD; $P < 0.001$ (**).

indeed activate transcription dependent on Jun phosphorylation. For this purpose we used stable transfectants of NIH·3T3 fibroblasts expressing the human CD53 antigen. This cell line was used instead of 293T cells, because 293T cells are very sensitive to the starvation used prior to the activation assay for the purpose of reducing the endogenous level of active kinase. For the transcription assays we used as targets of the CD53 activated-JNK a Gal4-c-Jun (1–223) and Gal-c-Jun (1–223, S63/73A, not phosphorylatable) fusion proteins and 5xGal4-Luc as reporter plasmid. To reduce background activity of the endogenous kinase, the cells were starved overnight. The cells were stimulated by addition of MEM53 for 10 min followed by a 6-h incubation to allow for transcription and translation of the reporter gene. Alternatively the cells were left in the presence of the antibody for the complete length of this period. In both cases the result was the same. As shown in Fig. 7, the ligation of the CD53 expressing cells, but not the control cells stably transfected with the empty vector, pMEXneo, resulted in activation of the luciferase activity if the wild-type Gal4-jun construct was used. But if the nonphosphorylatable double mutant (Gal-c-Jun S63/73A) was used, there was no activation of transcription. The background of activity in the cells transfected with empty vector is due to the remaining endogenous activity.

DISCUSSION

The information about the implication of tetraspanin antigens in cellular signalling is very limited, and is related mostly to their role as costimulatory molecules. CD53, like other tetraspanin antigens, has a costimulatory role in different cellular systems. CD9, CD81, CD82 and CD53 can have a costimulatory effect, with CD3, in interleukin-2 production in T-cells and Jurkat cells [10,22]. These costimulatory effects of tetraspanin proteins have been related to their physical association with other membrane proteins. But of the pathways implicated have not been identified from the tetraspanin perspective. All of them have been studied as a consequence of the physical interaction with each other, with integrins, or with growth factor receptors. The strength of these protein–protein interactions is different, as shown by the sensitivity of the membrane protein complexes to detergents [15]. Thus, the interactions of CD81 and CD151 are stronger than those of CD53, CD9 or CD37 [15]. However, despite the knowledge of some of the effects induced by tetraspanin proteins and the proteins with which they interact, the identification of the signalling pathways responsible for the biological effects have not yet been characterized.

Some of the biological effects induced by tetraspanin antigen ligation are observed in the absence of any additional costimulation. Ligation of CD53 antigen has been shown to induce homotypic adhesion [10], and also to activate or inhibit cell proliferation [10,55] depending on the mAb used. Two of the antibodies used in this work, 161-2 and 202-24B (Fig. 5), reduced cell proliferation by 70% in T cells [55]. Ligation of human CD53 with other antibodies, such as MEM53, has been shown to induce initiation of the G_1 phase of the cell cycle [23]. In that case additional signals are required to complete progression through the cell cycle. The differences in the effects caused by mAbs are due to their recognition of different epitopes on the CD53 molecule. All of these data indicated that tetraspanin proteins, or at least CD53, can have a signalling role by themselves. Natural ligands of CD53 or other tetraspan proteins are not yet known. In a way tetraspanin proteins can be considered as orphan receptors, and consequently because of that, almost all of their effects have been interpreted from the point of view of costimulatory roles.

It is clear that ligation of CD53 antigen induces *de novo* gene expression, such as the inducible nitric oxide synthase in macrophages [25], and thus the signal reaches the cell nuclei. This effect is mediated partly by PKC, because CD53 ligation induces translocation of this kinase to the cell membrane and it is sensitive to its inhibitors [25]; later the physical association between tetraspan proteins and PKC was demonstrated [38] which necessarily has to be a secondary event following translocation of PKC to the inner side of the plasma membrane, and thus is likely to be a consequence of the diacylglycerol induced by tetraspanin antigens [25,39]; however, nothing is yet known on further downstream components for the signals that originate in a tetraspanin antigen.

In this report we have shown that ligation of CD53 antigen, in rat and human cells, as well as in transfected cells, is able to trigger a three- to fourfold, quick and transient activation, of both endogenous and exogenous (transfected) JNK phosphorylation, which is independent of other membrane proteins, as suggested by its detection in very different cell types. This was demonstrated by phosphorylation of two of its substrates, Jun and ATF-2, as

fusion proteins. JNK activation has been related to many different biological effects, such as cell proliferation, differentiation and apoptosis, as well as the cellular response to stress [43]. The signals related to growth are transient and fast, whereas signals related to stress are slower in taking place, a consequence of its dependence on *de novo* protein synthesis. The phosphorylation of JNK, independent of Vav, in response to CD53 ligation might be a contributing pathway to cellular stimulation by other antigens, such as CD3 [52,56]. The independence of the activation from mediation by the *vav* oncogene, is consistent with the detection of this effect in a heterogeneous group of cell types, B and T cells, fibroblasts and carcinoma cells, as Vav is a signalling molecule that is implicated mainly in lymphocyte signalling [45].

The JNK pathway is activated in the cells as part of the response to many different types of signals, such as inflammatory cytokines [42], growth factors and activated oncogenes [57] that might have different outcomes ranging from development to apoptosis [41]. The activation of JNK activity by CD53 antigen ligation by itself, in the absence of cross-linking as shown in this report, indicates that this tetraspan antigen can modulate or cooperate with other cellular mechanisms that exert their effect via JNK, but that does not implicate a specific physical protein interaction of the CD53 antigen on the cell membrane. Thus CD53 antigen ligation can modulate a variety of processes, several of which are independent of the physical protein–protein interactions of CD53 on the membrane, such as the modulation of effects triggered by integrins or MHC class II antigens. The JNK pathway can provide a link between tetraspan antigens and their role as modulators of cell motility [58] and adhesion [59], processes modulated by signals converging on JNK activation [58,59].

The types of protein–protein interactions that tetraspanin antigens maintain on the cellular membrane are very heterogeneous. Therefore, it is likely that the intrinsic potential that CD53 antigen ligation, as a signal modulator, has on the JNK pathway could be enhanced or inhibited depending on the specific protein–protein association occurring in a particular type of cell. Thus, the transient activation of JNK by CD53 antigen ligation might have different biological consequences depending on the cell type and the other costimulatory signals that the cell is receiving. For example, strong immune challenge of T-cells does not require the activation of JNK, however, this activation is necessary for efficient responses in the presence of weak antigenic stimulation [60], a situation where CD53 and CD3 might stimulate JNK by different routes [44]. Furthermore, the observation that CD53 ligation triggers a response by the JNK indicates that CD53 signalling can also cooperate with other membrane proteins without the need for a specific physical CD53–protein interaction on the membrane, thus expanding its role as a costimulatory molecule. In this context, the role of CD53 as a stimulator of JNK activation might be important for adequate responses to a variety of other membrane receptors.

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