FEBS Letters 546 (2003) 218–222 FEBS 27362

Protein kinase CK2 phosphorylates the Fas-associated factor FAF1 in vivo and influences its transport into the nucleus

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Received 10 March 2003; revised 3 May 2003; accepted 3 May 2003

First published online 27 May 2003

Edited by Ulrike Kutay

Abstract We previously identified the Fas-associated factor FAF1 as an in vitro substrate of protein kinase CK2 and determined Ser289 and Ser291 as phosphorylation sites. Here we demonstrate that these two serine residues are the only sites phosphorylated by CK2 in vitro, and that at least one site is phosphorylated in vivo. Furthermore, we analyzed putative physiological functions of FAF1 phosphorylation. The ability of FAF1 to potentiate Fas-induced apoptosis is not influenced by the FAF1 phosphorylation status; however, the nuclear import of a phosphorylation-deficient FAF1 mutant was delayed in comparison to wild-type FAF1.

Key words: Fas-associated factor; FAF1; protein kinase CK2; Phosphorylation; Apoptosis; Nuclear translocation

1. Introduction

FAF1 was identified as a Fas-associated factor. Its function has been described as potentiating Fas-induced apoptosis in a mouse fibroblast cell line (L cells) and in a human T-cell tumor line (Jurkat) [1]. However, Ryu et al. [2] reported that upon overexpression in BOSC23 cells it can initiate apoptosis in the absence of an extrinsic death signal. FAF1 is widely expressed in different adult and embryonic tissues and in tumor cell lines [3,4]; it localizes to the cytoplasm and to the nucleus [3,5]; it contains domains also present in proteins of the ubiquitination pathway and a chromatin assembly factor-like domain [4,6,7]. FAF1 seems to function as a membrane-associated factor and as a nuclear factor. Roles in apoptosis, in ubiquitin-related processes, during embryonic differentiation and in tumorigenesis have been suggested.

The mechanisms by which FAF1 exerts its different functions are not known. Proteolytic processes might be of importance. A putative 40 kDa degradation product of FAF1 has been found in various human cell lines [4]. We have found after expression of recombinant FAF1 in *Escherichia coli*, spontaneously occurring 40 kDa degradation products comprising amino acids 1–313 and 1–315 [8]. Interestingly, an amino-terminal fragment of quail FAF1, FqFAF$^{M1-G467}$ caused apoptosis in COS cells [3]. The significance of the 40 kDa degradation product and its function remain to be investigated. Another regulatory mechanism might be through posttranslational modifications. We have previously identified FAF1 as an interacting partner of the regulatory β-subunit of the pleiotropic protein kinase CK2 [9] and as an in vitro substrate of CK2 [8]. We have determined two serine residues, namely serines 289 and 291, as in vitro phosphoacceptor sites. Here, we investigated whether the two identified serine residues were the only possible sites being phosphorylated by CK2 and whether these sites were phosphorylated in vivo. In order to find a physiological function for the CK2-mediated FAF1 phosphorylation, we analyzed the ability of FAF1 to potentiate Fas-induced apoptosis by using a phosphorylation-deficient FAF1 mutant. Since the phosphorylation sites Ser289 and Ser291 are located within the nuclear localization signal, we also investigated the subcellular localization of wild-type and mutant FAF1.

2. Materials and methods

2.1. Generation of FAF1 constructs

The coding region of human FAF1 was cloned into the vectors pQE30 (Qiagen), pEGFP N1 (Clontech) and pcDNA3.1/Myc-His(+) C (Invitrogen). Mutant FAF1 constructs FAF1^{A289}, FAF1^{A291} and FAF1^{A289,291} with serines at positions 289 and/or 291 exchanged for alanine were obtained using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

2.2. In vitro phosphorylation assay

Wild-type and mutant FAF1 constructs in the vector pQE30 were used for bacterial expression in the bacterial strain M15[pREP4]. Proteins were partially purified by a nickel nitriloacetic acid metal affinity chromatography and used for in vitro phosphorylation using recombinant CK2 holoenzyme as described previously [8].

2.3. Preparation of FAF1 for matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS)

COS-1 cells were transfected with the wild-type FAF1 construct in pcDNA3.1/Myc-His(+) C using the FuGENE™ 6 transfection reagent (Roche). 48 h after transfection cells were mechanically harvested and suspended in 25 mM Tris/HCl, pH 8.5, 1 mM dithiothreitol (DTT), 100 mM NaCl containing 1× Protease inhibitor cocktail (Roche). A lysate was prepared by sonication and cleared by centrifugation for 20 min at 4°C and 12 000 rpm (Heraeus Megafuge 1.0). The supernatant of a lysate from cells grown on a 10 cm plate was immunoprecipitated using 2 μg of anti-c-Myc antibody (sc-40, Santa Cruz, CA, USA). Altogether protein lysates from eight 10 cm plates, each transfected with 10 μg pcDNA3.1/Myc-FAF1 plasmid DNA were immunoprecipitated. After separation on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the protein band containing FAF1 was in-gel digested with trypsin, and subdigested with chymotrypsin

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00575-1

as previously described [8,10]. 0.1 M acetic acid was added and the supernatant used for further IMAC (immobilized metal ion affinity chromatography) and subsequent MALDI-MS analyses.

2.4. IMAC and MALDI-MS analyses

IMAC was used to enrich phosphopeptides [11]. The Ni-NTA-silica used for the IMAC column was charged with Fe^{3+} ions prior to use. A 2 cm long column was prepared in a 10 μl GELoader Tip (Eppendorf), washed twice with 20 μl 0.1 M acetic acid and loaded with the sample, followed by two washing steps with 20 μl 0.1 M acetic acid. Peptides were eluted with either 1 μl DHB matrix (H_2O/100% acetonitrile 1:1) directly on MALDI-MS target and subjected to MALDI-MS analysis, or with 1 μl ammonium solution, pH 10.5, into 0.5 μl 50 mM NH_4HCO_3 placed on target and dephosphorylated before MALDI-MS analysis. Dephosphorylation was done with 1 μl alkaline phosphatase (0.2 U/μl, 50 mM NH_4HCO_3, pH ≥ 7.8, Roche) on-target for 30 min at 37°C in a wet chamber. The dephosphorylated sample was mixed with 0.5 μl DHB matrix and allowed to dry before MALDI-MS analysis. Peptides were analyzed in a Bruker REFLEX MALDI time-of-flight mass spectrometer (Bruker-Daltonics) in positive reflector mode and positive ion linear mode. The ion acceleration voltage was set to 20 kV.

2.5. Transfection and Fas-induced apoptosis

Jurkat cells were transiently transfected with the wild-type or mutant FAF1 constructs in pEGFP. 30 h after transfection cells were incubated with 500 ng/ml of a monoclonal anti-human Fas antibody (SM1/1, Chemicon) for 30 min at 4°C and rinsed with prewarmed medium. Fas-mediated apoptosis was induced by addition of 5 μg/ml secondary crosslinking antibody (alkaline phosphatase-conjugated goat anti-mouse, Jackson Immunoresearch Laboratories). 16 h after crosslinking the cells were stained with either trypan blue (Gibco BRL) or with Hoechst 33342 (Sigma).

2.6. Subcellular localization

COS-1 cells grown on coverslips were transiently transfected with wild-type or mutant FAF1 constructs in the vector pEGFP N1. 24 or 48 h after transfection cells were harvested. Before direct fluorescence microscopy cells were incubated 20 min in the dark at room temperature with Hoechst 33342 at a final concentration of 1 ng/ml. 100 cells for each time point and transfection were analyzed by visual inspection.

3. Results and discussion

3.1. Ser289 and 291 are the only sites in the FAF1 protein phosphorylated in vitro by CK2

We have previously identified the residues Ser289 and Ser291 of the human FAF1 as in vitro CK2 phosphorylation sites [8]. To determine if there are other CK2 phosphorylation sites besides serines 289 and 291 within the FAF1 protein we used recombinant FAF1 mutants with either one or both serines exchanged for alanine for in vitro phosphorylation using recombinant CK2 holoenzyme. The phosphorylated proteins were resolved by SDS–PAGE, stained with Coomassie blue (Fig. 1A) and visualized by autoradiography (Fig. 1B). The amount of incorporated phosphate per mole FAF1 was calculated based on quantification of the radioactive bands (Fig. 1C). Wild-type FAF1 was phosphorylated to a greater extent than the single FAF1 mutants. Incorporation of phosphate was up to 1.3 mol/mol for wild-type FAF1 indicating phosphorylation of more than one site, whereas mutation of one of the two serines decreased the amount of incorporated phosphate to approximately half, suggesting that both serines are phosphorylated by recombinant CK2. No phosphorylation was observed in the $FAF1^{A289,291}$ double mutant. This demonstrates that phosphorylation of FAF1 by recombinant CK2 occurs at only these two previously identified residues, serine 289 and serine 291.

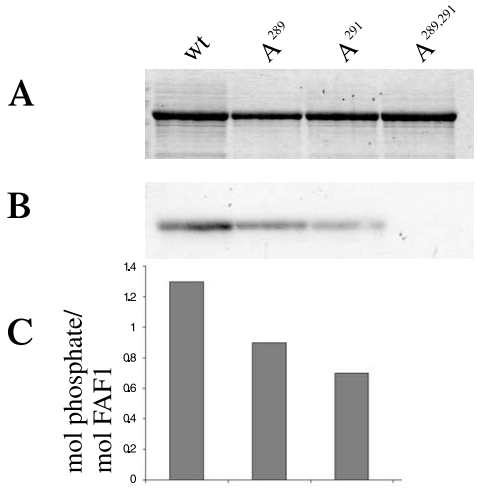

Fig. 1. In vitro phosphorylation of FAF1 constructs by recombinant CK2 holoenzyme. A: Coomassie blue staining of recombinant wild-type (wt) FAF1 constructs and constructs with amino acid exchanges at positions 289 and/or 291 after an in vitro phosphorylation reaction and SDS–PAGE. B: Corresponding autoradiography displaying the radioactively labeled FAF1 protein. C: Quantification of the radioactively labeled phosphate incorporated into the FAF1 protein.

3.2. Identification of the in vivo phosphorylation site in human FAF1

Next we wanted to know whether FAF1 is also phosphorylated in vivo. We transfected COS-1 cells with a wild-type human FAF1 expression construct, and immunoprecipitated FAF1 from protein extracts, followed by separation on a SDS–PAGE and in-gel digestion of FAF1 with trypsin. A small fraction of the supernatant containing the tryptic peptides was analyzed by MALDI-MS and thereby the identity of FAF1 was confirmed (data not shown). Peptides were then subdigested with chymotrypsin and phosphopeptides were enriched by an IMAC column. Analysis by MALDI-MS was carried out before (Fig. 2A) and after dephosphorylation (Fig. 2B).

The analysis of the phosphopeptide revealed phosphorylation of one peptide. The peptides at *m/z* 3974.5 and *m/z* 3990.2 correspond to the amino acid residues E276–F310, both containing one phosphorylated serine. The latter peptide contains an oxidized methionine and has therefore a correspondingly higher molecular mass. A third peak, barely above background at *m/z* 3893.8 corresponds to the dephosphorylated peptide (Fig. 2A). After dephosphorylation MALDI-MS analysis displayed two peptides at *m/z* 3894.5 and *m/z* 3910.9 corresponding to the amino acid residues E276–F310, with one oxidized methionine in the latter (Fig. 2B). The mass difference before and after dephosphorylation is 80 Da, which corresponds to a single phosphate group. This shows that one residue in the peptide E276–F310 is phosphorylated in vivo. The same phosphopeptide E276–F310 had been identified after in vitro phosphorylation of FAF1 by CK2 [8].

The sequence of the chymotryptic peptide E276–F310 is shown in Fig. 2C. It contains two threonine and three serine residues, two of which are located in a bona fide CK2 consensus sequence which requires an acidic residue at position +3 [12], i.e. serines 289 and 291. This fact together with our finding that CK2 is unable to phosphorylate the $FAF1^{A289,291}$

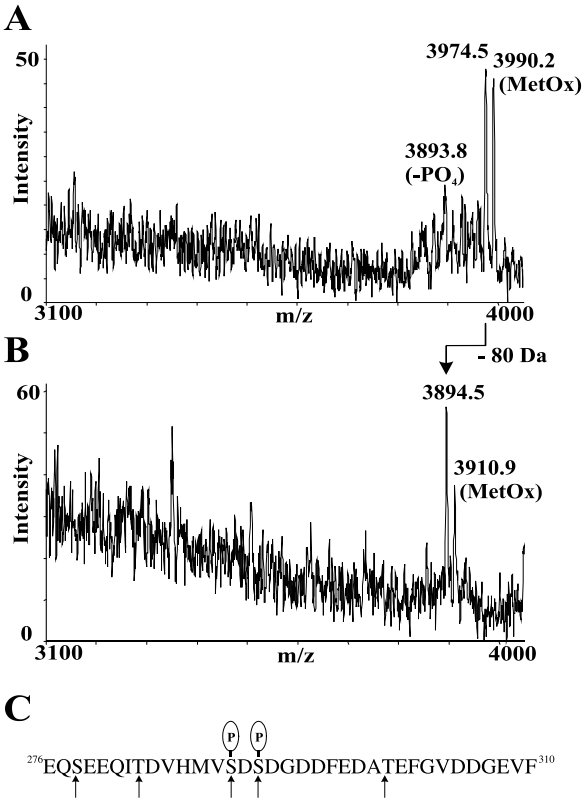

Fig. 2. Identification of the in vivo phosphorylation site in FAF1. Tryptic/chymotryptic FAF1 phosphopeptides were isolated using nanoscale FE(III) IMAC and analyzed by MALDI-MS. A: A single peptide was detected carrying one phosphate group (*m/z* 3974.5). B: After treatment with alkaline phosphatase, the mass of the major peptide decreased by 80 Da (*m/z* 3894.5). C: The sequence of the chymotryptic peptide 276–310 from FAF1. Serines and threonines are indicated by arrowheads, the two possible CK2 phosphorylation sites are indicated by attached phosphate groups (P).

mutant in vitro implies that S289 and/or 291 are also phosphorylated in vivo. However, it could not be discerned which of the two serines was phosphorylated. Since the two serines are located so close together one might speculate that they could easily substitute one another and are phosphorylated equally well, as it has been shown in vitro.

3.3. CK2-mediated FAF1 phosphorylation does not affect potentiation of Fas-induced apoptosis

The only so far described physiological property of FAF1 is to be a positive regulator of apoptosis [1,2]. Chu et al. demonstrated that FAF1 potentiates Fas-induced apoptosis. To examine whether phosphorylation of FAF1 by CK2 could affect this property, a transient apoptosis assay was performed. Wild-type FAF1 and FAF1^{A289,291} were transiently overexpressed in Jurkat cells. This cell line was chosen, since it is responsive to Fas-induced apoptosis. Apoptosis was induced by treatment with an anti-Fas antibody followed by crosslinking with a secondary antibody. Dead and apoptotic cells were visualized by trypan blue staining (Fig. 3A) and Hoechst staining (Fig. 3B), respectively.

The wild-type FAF1 construct led to more dead and more apoptotic cells upon treatment with the anti-Fas antibody than it was observed in mock-transfected cells (Fig. 3). 24% of control cells were apoptotic when treated with the anti-Fas antibody, whereas this percentage increased to approximately 60% after transfection with the wild-type FAF1 construct. Fas-induced apoptosis was neither increased nor decreased by the phosphorylation-deficient FAF1 mutant FAF1^{A289,291} in comparison to wild-type FAF1, indicating that phosphorylation of FAF1 by CK2 does not affect potentiation of Fas-induced apoptosis. However, a role for CK2 during apoptosis has recently emerged [13] and for a number of proteins CK2-mediated phosphorylation has an anti-apoptotic function, e.g. phosphorylation of HS1 [14], Max [15], Bid [16] and connexin 45.6 [17] by CK2 prevents their cleavage by caspases and ARC when phosphorylated by CK2 is targeted to mitochondria where it inhibits caspase 8 [18].

It should be noted that transfection of FAF1 alone without inducing Fas-mediated apoptosis did not cause apoptosis in Jurkat cells (Fig. 3). Also overexpression of the phosphorylation-deficient FAF1^{A289,291} mutant alone was not sufficient to induce apoptosis (data not shown). Interestingly, in BOSC23 cells mere overexpression of FAF1 was sufficient to initiate apoptosis, and beside the UB2 domain of FAF1 also the nuclear localization sequence which contains the CK2 phosphorylation sites was required [2]. Yet, neither in Jurkat cells, as shown here, nor in L cells [1] or NIH3T3 fibroblasts and COS-7 cells [3], enhanced FAF1 expression led to apoptosis.

3.4. CK2-mediated phosphorylation of FAF1 influences its nuclear localization

The FAF1 phosphorylation sites serine 289 and 291 are located in a domain covering the residues 275–312 of the human sequence. This domain has been shown to be responsible for nuclear localization [3]. Therefore we analyzed the effects of CK2-mediated phosphorylation on the cellular local-

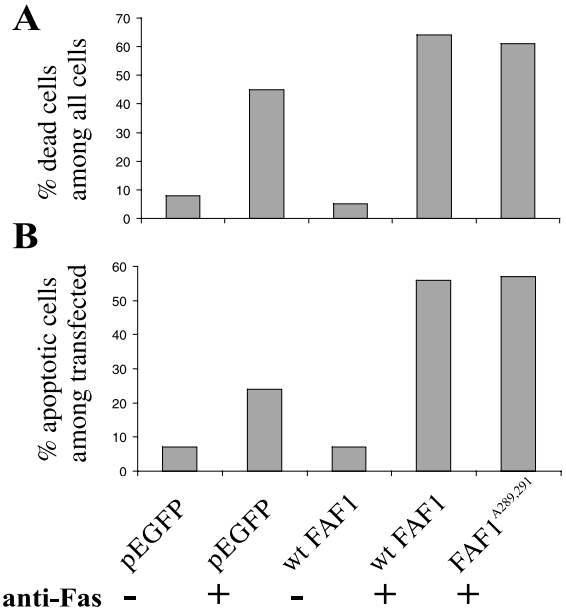

Fig. 3. Influence of wild-type FAF1 and a phosphorylation-deficient FAF1 mutant on Fas-induced apoptosis. Jurkat cells were transfected with the empty vector (pEGFP), wild-type (wt) FAF1 or mutant FAF1^{A289,291} constructs in the vector pEGFP followed by anti-Fas antibody crosslinking as described in Section 2. A: Dead cells were counted after staining with trypan blue. B: Cells were stained with Hoechst and apoptotic cells were counted among transfected cells using fluorescence microscopy.

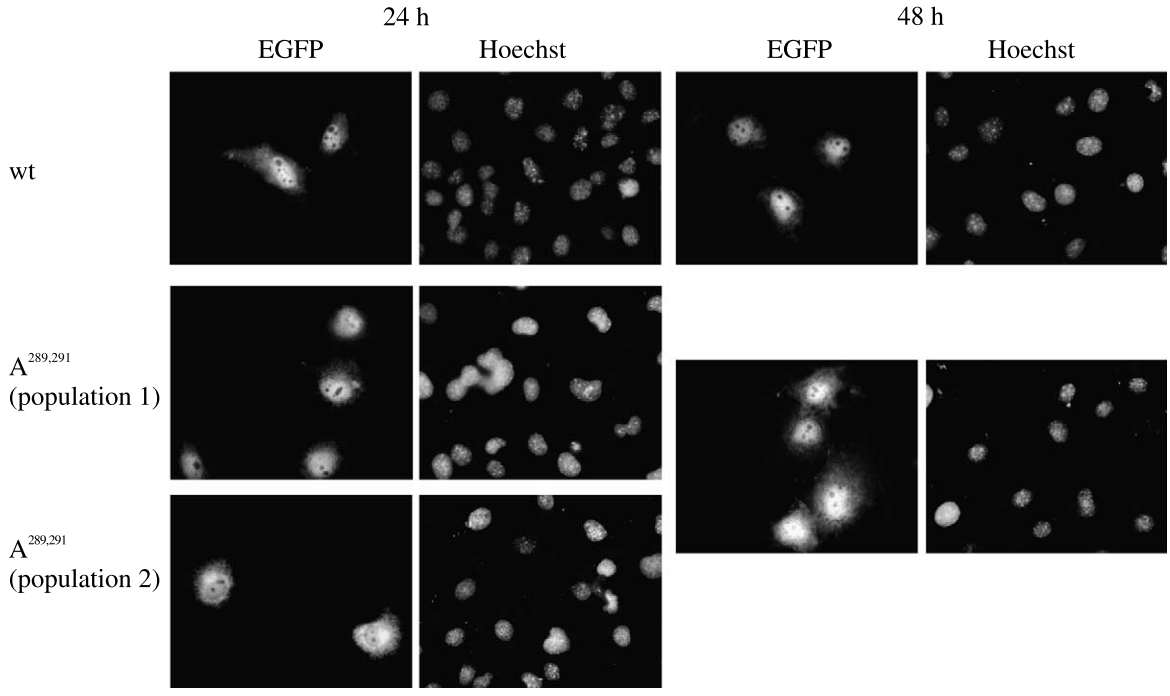

Fig. 4. Subcellular localization of wild-type FAF1 and a phosphorylation-deficient FAF mutant. COS-1 cells were transfected with wild-type (wt) FAF1 or mutant FAF1^{A289,291} constructs in the vector pEGFP and analyzed by fluorescence microscopy for EGFP and parallel Hoechst staining 24 and 48 h (h) after transfection. The pictures shown for FAF1^{A289,291}, population 1 and population 2, 24 h after transfection represent 70 and 30% of the cells, respectively.

ization of FAF1. COS-1 cells were transiently transfected with wild-type FAF1 or FAF1^{A289,291} constructs, tagged with EGFP. 24 and 48 h after transfection, localization of FAF1 was analyzed directly by immunofluorescence microscopy (Fig. 4).

Transfection with wild-type FAF1 resulted in a predominantly nuclear localization. FAF1 was distributed homogeneously over the nuclei but excluded from the nucleoli 24 and 48 h after transfection (Fig. 4). By contrast the FAF1 mutant FAF1^{A289,291}, which cannot be phosphorylated, showed a different localization 24 h after transfection. Two cell populations were detected. In approximately two third of the cells (population 1) the FAF1 mutant showed the same cellular localization as the wild-type FAF1, whereas in about one third of the cells (population 2) a predominantly cytoplasmic, perinuclear staining was observed. 48 h after transfection with FAF1^{A289,291} all cells had the protein predominantly in the nucleus, but it was excluded from the nucleoli. The results were the same for proteins tagged with a myc epitope instead of EGFP and analyzed by immunostaining (not shown). By using anti-FAF1 antibodies the described results were also confirmed (not shown).

Although it is known that the cell cycle and/or the state of confluency can influence the phosphorylation state of proteins and their cellular localization, wild-type FAF1 was, in all cells analyzed, predominantly found in the nucleus. Since CK2 is constitutively active during all stages of the cell cycle [19], one might speculate that FAF1 is phosphorylated all the time. 30% of the cells transfected with a phosphorylation-deficient FAF1 mutant did not show the predominant nuclear localization. Therefore we assume that CK2-mediated FAF1 phosphorylation influences the nuclear localization of FAF1. It seems that abrogation of phosphorylation does not completely inhibit the nuclear transport, but slows down the import of FAF1 into the nucleus.

The predominant nuclear localization of FAF1 and exclusion from the nucleoli is in agreement with results from Frohlich et al. [3]. However, it implies that the major function of FAF1 might not be in the cytoplasm as an interacting partner of Fas. The accumulation of FAF1 in the nucleus is obviously regulated by several means, among them phosphorylation by CK2. But phosphorylation is not an exclusive signal as it is the case for many other proteins. E.g. phosphorylation of the large T-antigen by CK2 increases the rate of nuclear import from 10 h for a phosphorylation-deficient T-antigen mutant to 15–20 min when the T-antigen is phosphorylated by CK2 [20]. Also the nuclear import of IFI 16 and of nucleoplasmin are regulated by CK2 phosphorylation [21,22]. Current models for mechanisms of regulation of nuclear transport also involve ubiquitination [23], which was shown to be necessary for transport of NF-κB across the nuclear membrane. Further studies are necessary to reveal whether the ubiquitin binding domain and the ubiquitin-like domains of FAF1 do have a contribution in this respect. In mouse models for cardiac hypertrophy, susceptible for apoptosis but not showing apoptosis, high levels of FAF1 were detected, supporting the notion of FAF1 not only being confined to Fas association but also being involved in other yet unknown functions [24]. Hence, special importance will be to elucidate the nuclear function of FAF1.

Acknowledgements: Preliminary results were obtained by A. Kolding, C.W. Yde and J.C. Kristensen during their Bachelor projects. We thank Hans H. Jensen for expert technical assistance and Dr. S. Douthwaite for critically reading the manuscript. O.G.I. is supported by the Danish Cancer Society (grant no. 96 100 40) and the Danish Research Council (grant no. 9601695).

References

[1] Chu, K., Niu, X. and Williams, L.T. (1995) Proc. Natl. Acad. Sci. USA 92, 11894–11898.
[2] Ryu, S.W. and Kim, E. (2001) Biochem. Biophys. Res. Commun. 286, 1027–1032.
[3] Frohlich, T., Risau, W. and Flamme, I. (1998) J. Cell. Sci. 111, 2353–2363.
[4] Ryu, S.W., Chae, S.K., Lee, K.J. and Kim, E. (1999) Biochem. Biophys. Res. Commun. 262, 388–394.
[5] Guerra, B., Boldyreff, B. and Issinger, O.G. (2001) Int. J. Oncol. 19, 1117–1126.
[6] Becker, K., Schneider, P., Hofmann, K., Mattmann, C. and Tschopp, J. (1997) FEBS Lett. 412, 102–106.
[7] Buchberger, A., Howard, M.J., Proctor, M. and Bycroft, M. (2001) J. Mol. Biol. 307, 17–24.
[8] Jensen, H.H., Hjerrild, M., Guerra, B., Larsen, M.R., Højrup, P. and Boldyreff, B. (2001) Int. J. Biochem. Cell Biol. 33, 577–589.
[9] Kusk, M., Ahmed, R., Thomsen, B., Bendixen, C., Issinger, O.G. and Boldyreff, B. (1999) Mol. Cell. Biochem. 191, 51–58.
[10] Kussmann, M., Nordhoff, E., Rahbek-Nielsen, H., Haebel, S., Rossel-Larsen, M., Jakobsen, L., Gobom, J., Mirgoroskaya, E., Kroll-Kristensen, A., Palm, L. and Roepstorff, P. (1997) J. Mass Spectrom. 32, 593–601.
[11] Stensballe, A., Andersen, S. and Jensen, O.N. (2001) Proteomics 1, 207–222.
[12] Meggio, F., Marin, O. and Pinna, L.A. (1994) Cell. Mol. Biol. Res. 40, 401–409.
[13] Ahmed, K. and Gerber, D.A. (2002) Trends Cell Biol. 12, 226–230.
[14] Ruzzene, M., Penzo, D. and Pinna, L.A. (2002) Biochem. J. 364, 41–47.
[15] Krippner-Heidenreich, A., Talanian, R.V., Sekul, R., Kraft, R., Thole, H., Ottleben, H. and Luscher, B. (2001) Biochem. J. 358, 705–715.
[16] Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, E., Hochmann, A., Journot, L., Antonsson, B. and Martinou, J.C. (2001) Mol. Cell 8, 601–611.
[17] Yin, X., Gu, S. and Jiang, J.X. (2001) J. Biol. Chem. 276, 34567–34572.
[18] Li, P.F., Li, J., Mueller, E.C., Otto, A., Dietz, R. and von Harsdorf, R. (2002) Mol. Cell 10, 247–258.
[19] Schmidt-Spaniol, I., Grimm, B. and Issinger, O.G. (1993) Cell. Mol. Biol. Res. 39, 761–772.
[20] Huebner, S., Xiao, C.Y. and Jans, D.A. (1997) J. Biol. Chem. 272, 17191–17195.
[21] Briggs, L.J., Johnstone, R.W., Elliot, R.M., Xiao, C.Y., Dawson, M., Trapani, J.A. and Jans, D.A. (2001) Biochem. J. 353, 69–77.
[22] Vancurova, I., Paine, T.M., Lou, W. and Paine, P.L. (1995) J. Cell Sci. 108, 779–787.
[23] Karin, M. and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663.
[24] Aronow, B.J., Toyokawa, T., Canning, A., Haghighi, K., Delling, U., Kranias, E., Molkentin, J.D. and Dorn II, G.W. (2001) Physiol. Genomics 6, 19–28.