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Adhesion induced expression of the serine/threonine kinase *Fnk* in human macrophages

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Members of the *polo* subfamily of protein kinases play crucial roles in cell proliferation. To study the function of this family in more detail, we isolated the cDNA of human *Fnk* (FGF-inducible kinase) which codes for a serine/threonine kinase of 646 aa. Despite the homology to the proliferation-associated *polo*-like kinase (*Plk*), tissue distribution of *Fnk* transcripts and expression kinetics differed clearly. In contrast to *Plk* no correlation between cell proliferation and *Fnk* gene expression was found. Instead high levels of *Fnk* mRNA were detectable in blood cells undergoing adhesion. The transition of monocytes from peripheral blood to matrix bound macrophages was accompanied by increasing levels of *Fnk* with time in culture. Neither treatment of monocytes with inducers of differentiation nor withdrawal of serum did influence *Fnk* mRNA levels significantly, suggesting that cell attachment triggers the onset of *Fnk* gene transcription. The idea that Fnk is part of the signalling network controlling cellular adhesion was supported by the analysis of the cytoplasmic distribution of the *Fnk* protein and the influence of its overexpression on the cellular architecture. *Fnk* as fusion protein with GFP localized at the cellular membrane in COS cells. Dysregulated *Fnk* gene expression disrupted the cellular f-actin network and induced a spherical morphology. Furthermore, *Fnk* binds to the Ca^{2+}/integrin-binding protein *Cib* in two-hybrid-analyses and co-immunoprecipitation in assays. Moreover, both proteins were shown to co-localize in mammalian cells. The homology of *Cib* with calmodulin and with calcineurin B suggests that *Cib* might be a regulatory subunit of *polo*-like kinases. *Oncogene* (2000) **19,** 4832–4839.

Keywords: polo-like kinase; adhesion; macrophages; serine/threonine kinase

Introduction

Protein kinases are involved in controlling mitogenesis, cellular differentiation and changes of cellular structures. The Drosophila gene *polo* and the yeast cell cycle genes *Cdc5* and *plo1* encode related protein kinases which are required for progression through mitosis (Sunkel and Glover, 1988; Llamazares *et al*., 1991; Fenton and Glover, 1993; Kitada *et al*., 1993; Ohkura *et al*., 1995; Toczyski *et al*., 1997). In recent studies mammalian protein kinases have been identified which are homologous to the Drosophila gene *polo* (Simmons *et al*., 1992; Clay *et al*., 1993; Lake and Jelinek, 1993; Golsteyn *et al*., 1994; Hamanaka *et al*., 1994; Holtrich *et al*., 1994). To date the subfamily of *polo*-related protein kinases encompasses the murine kinases *Snk*, *Plk*, *Sak* and *Fnk* (Simmons *et al*., 1992; Clay *et al*., 1993; Fode *et al*., 1994; Donohue *et al*., 1995) as well as the human orthologues *Sak* and *Plk* (Lake and Jelinek, 1993; Golsteyn *et al*., 1994; Hamanaka *et al*., 1994; Holtrich *et al*., 1994; Karn *et al*., 1997). The expression of the *Plk* gene is tightly linked to cell proliferation and regulated during cell cycle progression (Clay *et al*., 1993; Lake and Jelinek, 1993; Holtrich *et al*., 1994). Several lines of evidence support an essential role of *Plk* in the control of cell cycle-dependent checkpoints during G_2 (Llamazares *et al*., 1991; Okhura *et al*., 1995; Kumagai and Dunphy, 1996). In mammalian cells *Plk* interacts with polypeptides which constitute the mitotic spindle and microinjection of *Plk*-specific antibodies resulted in a G_2-arrest (Golsteyn *et al*., 1995; Lee *et al*., 1995; Lane and Nigg, 1997).

Snk was the first protein kinase to be identified as mammalian member of this subfamily. It was classified as an immediate early gene by virtue of its increased mRNA expression upon stimulation with growth factors (Simmons *et al*., 1992). Similarly, murine *Fnk* was shown to be an immediate early gene as its expression is responsive to the addition of growth factors (Donohue *et al*., 1995). The *Fnk* protein, which was found in serum-starved cells, becomes phosphorylated at the beginning of the cell cycle by the addition of serum and is dephosphorylated at the exit of mitosis (Chase *et al*., 1998). The abundance of the *Fnk* protein increases as cells progress from G_1 to mitosis. The kinase activity correlates with the mitotic phosphorylation of *Fnk* (Chase *et al*., 1998), suggesting that the protein is involved in mitosis-specific signalling.

In this report we describe the cloning and genetic analysis of a *polo*-related human gene, which represents the human counterpart of murine *Fnk*. In contrast to Plk, Northern blot analyses revealed no correlation of human *Fnk* expression with the proliferative activity of cells. Instead *Fnk* transcript levels were upregulated during adhesion of human monocytes in culture. Dysregulated expression of *Fnk* induced a spherical

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cellular shape due to the disruption of the cellular f-actin network. We show that *Fnk* co-immunoprecipitates and co-localizes with the Ca^{2+}/integrin-binding protein *Cib*, previously identified as an *Fnk* interaction partner using the two-hybrid-system (Kauselmann *et al*., 1999). Our data suggest that beyond its role during cell cycle, *Fnk* is also part of the signalling network controlling cellular adhesion.

Results

PCR-based isolation of a Plk-*related protein kinase cDNA from human embryonic tissues*

cDNA from human embryonic tissues was amplified using degenerate primers corresponding to highly conserved aa motifs (WVXKWVDYS and DHTK-XIXC) from the *polo*-box of human *Plk* (Holtrich *et al*., 1994) in order to identify new *Plk*-related genes. One of the isolated clones (termed U1) representing an unknown gene was chosen for further studies because of its homology to *Plk*. In order to obtain the complete open reading frame, screenings of cDNA- and genomic-libraries as well as RACE-PCRs were performed. Determination of the nucleotide sequence of U1 revealed an open reading frame of 1938 nt extending from an ATG codon at position 240 to an in-frame stop codon at position 2178 predicting a 72 kDa-polypeptide of 646 aa. Comparing the kinase domain of U1 to those of *Plk* and *Snk* the similarity was determined to be 54.8 and 68.0%, respectively. A close overall homology of 92% was found to the entire open reading frame of murine *Fnk* (FGF-inducible kinase). Due to the high degree of homology to *Fnk*, it seems likely that U1 is the human counterpart of the murine gene *Fnk* (Donohue *et al*., 1995). Thus, we suggest to name it also *Fnk*. Li *et al*. (1996) described a human gene, called *Prk* (proliferation related kinase), which displays an aa homology of 100% compared to human *Fnk* (U1). However, our human *Fnk*-isolate revealed an extension of 39 aa at the aminoterminus indicating that the human and murine FNK proteins are almost identical in length (EMBL Bank Data AJ 293866).

All 3 *polo*-related proteins are characterized by the same domain topology and by specific motifs of this subfamily. The aa sequences of the catalytic domains of human serine/threonine kinases were used for the calculation of a phylogenetic tree with the Tree program of HUSAR (DKFZ, Heidelberg) (Feng and Doolittle, 1987). This comparison revealed that *polo*-related kinases diverged before the subfamily of Calmodulin kinase-related genes developed.

Comparative expression analysis of polo-*related kinases in human cells and adult tissues*

We started the study by performing a Northern blot analysis to compare the expression patterns of *Fnk* and other human *polo*-like kinases. *Fnk* transcripts exhibiting a length of 2.4 kb are most abundant in placenta and lung (data not shown). A lower frequency of *Fnk* transcripts was observed in skeletal muscle, heart, pancreas and kidney. Weak signals were detected in liver and brain. Rehybridizing the same blot with a probe specific for human *Snk* revealed a distribution of mRNA similar to that observed for *Fnk* (data not shown). *Snk*-specific transcripts of 2.9 kb were found in heart, brain, placenta, lung, liver, skeletal muscle and pancreas. In contrast to this broad tissue distribution of *Fnk*- and *Snk*-transcripts, *Plk* expression is restricted to proliferating tissues such as placenta, colon and testis (Holtrich *et al*., 1994; Golsteyn *et al*., 1995). In human blood cells, strong *Fnk* expression was observed only in terminally differentiated macrophages whereas both *Snk* and *Plk* expression were barely detectable in these cells. None of these three genes was expressed at considerable levels in resting lymphocytes isolated from peripheral blood (data not shown).

Fnk *expression is not regulated in proliferating A431 and lung tumour cells*

Expression of *Plk*, *Snk* and *Fnk* was induced in cells after addition of serum or growth factors (Simmons *et al*., 1992; Holtrich *et al*., 1994; Donohue *et al*., 1995). Furthermore, *Plk* expression correlated with the proliferative activity of cells (Holtrich *et al*., 1994). Here, we found that mRNA levels of *Fnk* were moderately reduced in rare lung tumours compared to surrounding non-affected tissues (Figure 1). Unlike results obtained with murine *Fnk* in NIH3T3 cells (Donohue *et al*., 1995) we were unable to show a significant change of human *Fnk* expression in A431 cells which were serum-starved and restimulated (data not shown). These observations indicate that despite the close relationship to *Plk*, transcript levels of *Fnk* do not correlate to the mitotic index of human cells.

Fnk *mRNA is differentially expressed in cells of hematopoietic origin*

Since we found *Fnk* to be strongly expressed in terminally differentiated macrophages but not in resting or activated lymphocytes (data not shown), we examined *Fnk* gene expression during hematopoietic

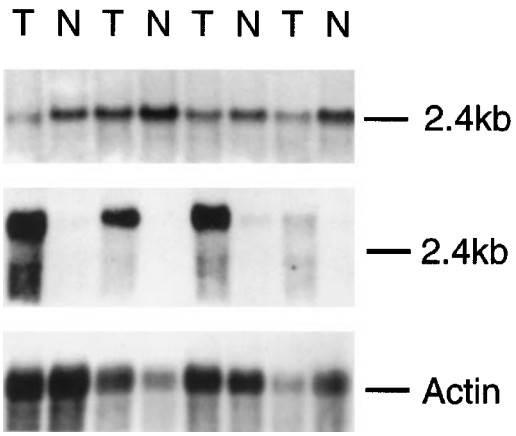

Figure 1 Expression of *Fnk* and *Plk* mRNA in human lung tumours. Each lane contained 20 μg of total RNA from human adult normal and malignant lung tissues. The blots were hybridized under high stringency conditions with antisense probes corresponding to aa 280–479 of *Fnk* (upper panel) and to aa 285–497 of *Plk* (lower panel). Lanes 1, 3, 5 and 7, squamous-cell carcinomas; lanes 2, 4, 6 and 8, corresponding surrounding tissues (normal lung). RNAs in lanes 1 and 2 (3 and 4; 5 and 6; 7 and 8) are from the same individual

cell differentiation in more detail. Human monocytes from peripheral blood were kept in culture to analyse *Fnk* mRNA induction profiles during macrophage development. As shown in Figure 2a (lanes 1-5) expression of *Fnk* was induced within one day in tissue culture dishes and increased with prolonged incubation proposing that the expression of *Fnk* correlates with an early stage of differentiation and/or cellular adhesion. Interestingly, hybridizing the same blot with a human *Snk* probe revealed an inverse pattern of expression. At the onset of *Fnk* gene activity *Snk* expression ceased (Figure 2a).

We next analysed whether the induction of *Fnk* is associated with the differentiation of monocytes to macrophages or rather is a consequence of adhesion. Transfer of peripheral monocytes to culture flasks and incubation in serum-free medium for one day was sufficient to induce strong *Fnk* expression (data not shown). Neither AB serum nor vitamin D_3, which are both inducers of macrophage differentiation, further increased *Fnk* expression (data not shown). Additional evidence for the independence of *Fnk* mRNA expression from the status of monocytic maturation came from experiments with the hematopoietic cell lines THP1, U937 and HL60 (Figure 2b). Under the influence of vitamin D_3 these cells develop a macrophage-like phenotype. The low expression of *Fnk* in these cell lines was independent of vitamin D_3. Different levels of *Fnk* expression in these cell lines compared to mature macrophages may rather be due to their different capability to adhere to tissue culture plastic. These results suggest that cellular adhesion rather than maturation triggers *Fnk* induction.

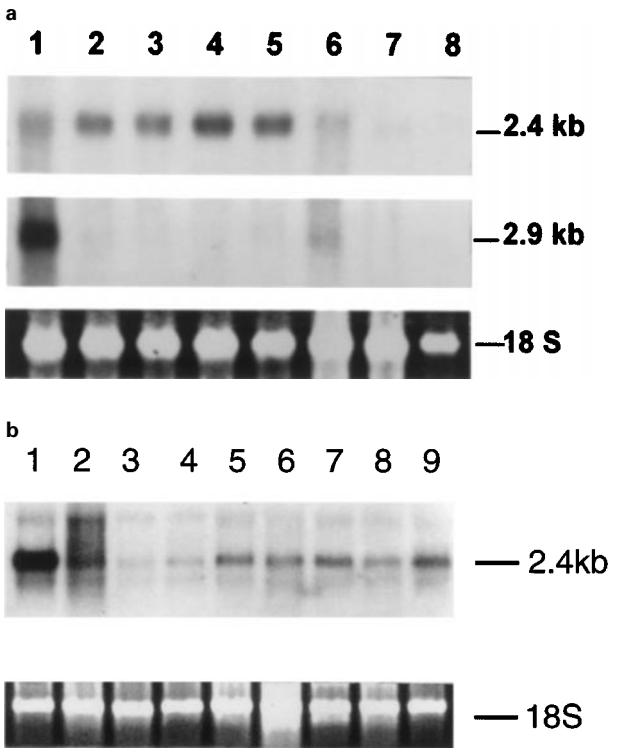

Figure 2 Expression of *Fnk* mRNA in human cells. Each lane contained 20 μg of total RNA from various human cells cultured in the presence of serum. The RNA was hybridized with probes corresponding to aa 390–499 of *Snk* and to aa 280–479 of *Fnk*. (**a**) Upper panel: *Fnk*, lower panel: *Snk*. Lanes 1–5, monocytes from human peripheral blood were cultured for 0, 1, 2, 4 and 8 days, lane 6, lymphocytes from peripheral blood, lane 7, myeloid cell line U937 and lane 8, primary human fibroblasts. (**b**) RNA was hybridized with the *fnk* probe as described above. Lane 1, monocytes cultured for 7 days in the presence of 10% fetal bovine serum, lane 2, monocytes from peripheral blood. Lanes 3–8: Cell lines were cultivated with (lanes 3,5 and 7) or without (lanes 4,6 and 8) vitamin D_3: THP1 (lanes 3,4), U937 (lanes 5,6), HL60 (lanes 7,8) and K562 (lane 9). The corresponding ethidium bromide-stained gel (with position of 18S rRNA) is shown below the autoradiograms to indicate the amount of RNA loaded

Transcript levels of Fnk *are upregulated upon adhesion of human monocytes*

We next investigated the influence of adherence on *Fnk* expression by analysing monocytes kept under non-adherent conditions. After 2, 8 and 18 h in culture expression of *Fnk* is clearly elevated in adherent monocytes (Figure 3, lanes 1, 4, 8 and 12) compared to non-adherent cells (Figure 3, lanes 2, 6 and 10). There was no change in induction of *Fnk* expression observed when the cells were treated with serum (Figure 3, lanes 3, 5, 7, 9 and 11). An exception was seen in monocytes cultured for 18 h, where we found the *Fnk* signal to be less intense in cells following serum-treatment (Figure 3, lanes 12 and 13).

The Fnk *protein is localized at the plasma-membrane*

We next analysed whether the distribution of *Fnk* in mammalian cells might be linked to cellular adhesion processes. To determine the intracellular distribution of *Fnk*, the expression of a *Fnk*-GFP fusion protein was analysed in COS cells. Expression of parental GFP resulted in a diffuse cytoplasmic and nuclear distribution. In contrast, fusion of GFP to *Fnk* altered the localization of the fluorescence in most COS cells from a diffuse to a distinct continuous pericytoplasmic staining at the cellular membrane (Figure 4a). In a low percentage of transfected cells the GFP-*Fnk* hybrid-protein was observed in patch-like regions at the cellular membrane (Figure 4b). The localization of

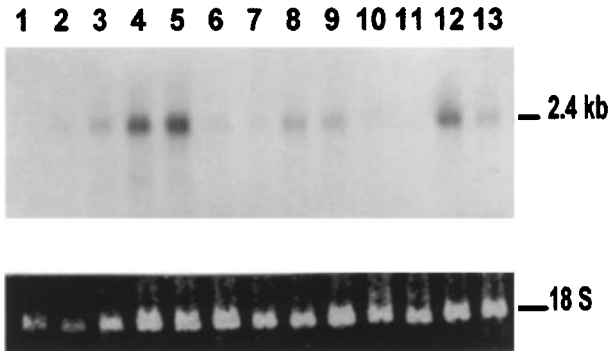

Figure 3 Expression of *Fnk* in primary human monocytes/macrophages under adherent and non-adherent conditions. Each lane contained 20 μg of total RNA which was hybridized with a *Fnk*-specific probe as described in Figure 1. Lane 1: monocytes from peripheral blood; lanes 2–5: monocytes were cultured for 2 h without serum, non-adherent (lane 2), with serum, non-adherent (lane 3), without serum, adherent (lane 4) and with serum, adherent (lane 5); lanes 6–9: monocytes were cultured for 8 h without serum, non-adherent (lane 6), with serum, non-adherent (lane 7), without serum, adherent (lane 8) and with serum, adherent (lane 9); lanes 10–13: monocytes were cultured for 18 h without serum, non-adherent (lane 10), with serum, non-adherent (lane 11), without serum, adherent (lane 12) and with serum, adherent (lane 13)

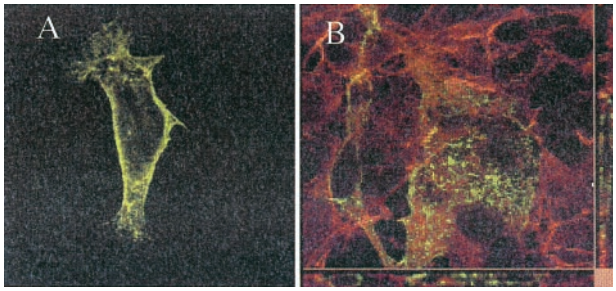

Figure 4 Cellular localization of *Fnk*. COS cells were transfected with an expression plasmid coding for GFP-fusion proteins with *Fnk*. Forty hours after transfection live cell fluorescence was determined by CLSM. (**a**) Optical section of a cell expressing GFP-labelled *Fnk* (green). The GFP-fluorescence highlights predominantly the cellular membrane. (**b**) Optical section of a GFP-*Fnk*-transfected COS cell culture stained for f-actin using TRITC-phalloidin (tetramethyl-rhodamin-isothiocyanate-coupled phalloidin) (red). Cells express GFP-labelled *Fnk* (green). In a low percentage of cells GFP-fluorescence is restricted to the cell membrane in a patch-like fashion. Both the upper and the substrate facing membrane exhibit the same fluorescence intensity. F-actin staining and GFP-*Fnk* are almost exclusive. The images on the right side and bottom are cross sections along a line marked by small triangles on the separating lines

Fnk differs clearly from either the diffuse cytoplasmic distribution of *Snk* or the patch-like localization of *Plk* (data not shown). In NIH3T3 fibroblasts the distribution of the GFP-hybrid-proteins fused to *Fnk*, *Plk* and *Snk* was similar as in COS cells (data not shown).

Fnk *overexpression disrupts the f-actin cytoskeleton component*

Serine-threonine kinases such as protein kinase C (PKC) and mitogen-activated protein (MAP) kinases are activated upon adhesive interactions, and inhibitors of PKC block cell attachment and spreading in certain cell systems (Chen *et al*., 1994; Keenan and Kelleher, 1998; Vuori and Ruoslahti, 1993). To evaluate the role of *Fnk* in these processes, the influence of its elevated activity was tested. After transfection two different phenotypes were observed. Cells exhibiting weak fluorescence (approximately 10% of transfectants) due to GFP-*Fnk* expression retained an almost typical COS cell shape (Figure 5a), while in cells with strong fluorescence a change from outspread to a spherical phenotype was visible (Figure 5c). Analysing the distribution of cytoskeletal components in those cells revealed that expression of GFP-*Fnk* disrupts the cellular f-actin cortex. A clear reduction of the cellular f-actin cortex was also observable in cells expressing low levels of GFP-*Fnk*-fluorescence (Figure 5b). However, comparing outspread and spherical cells the overall distribution of GFP-*Fnk* seemed to persist, suggesting that the kinase is not associated with submembraneous actin. *Fnk* remained evenly distributed along the cell membranes and no difference was found between the substrate facing and the non-adhering culture medium facing membrane. The assumption that *Fnk* is not bound to actin is in line with results obtained from experiments using cells expressing low levels of GFP-*Fnk* treated with the actin depolymerizing drug cytochalasin D (CD). A 30 min treatment with CD (1 ug/ml) disturbed the normal actin pattern. The f-actin cortex disappeared and small

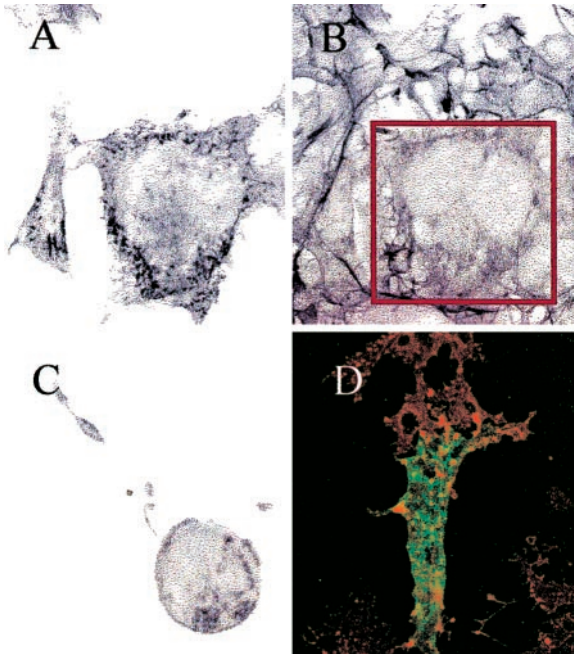

Figure 5 *Fnk* expression disrupts the f-actin cytoskeleton. Single optical section (by CLSM) taken through a COS cell culture stained for F-actin using TRITC-phalloidin. (**a**) Two cells expressing GFP-labelled *Fnk* (black) are shown. (**b**) The outline of one of the *Fnk*-positive cells shown in **a** is marked in the TRITC-phalloidin image (red rectangle). No brightly TRITC-fluorescing zones are found in the *Fnk*-positive cells (black). By contrast in non-*Fnk* expressing neighbouring cells several areas of strong TRITC-fluorescence are observable. This indicates reduction of F-actin in the GFP-*Fnk*-positive cells. (**c**) Influence of strong *Fnk* expression on the cellular morphology. Single optical section (taken by CLSM) of a GFP-*Fnk* expressing COS cell. The cell became almost spherical, *Fnk* is located mostly along the plasma membrane and in some intracellular granules. (**d**) Cellular distribution of f-actin in *Fnk*-expressing cells treated with cytochalasin D (1 μg/ml) for 30 min. Section close to the surface of the *Fnk*-positive cell: F-actin patches (red) are scattered in between the *Fnk*-positive (green) membrane domains

f-actin patches were formed (Figure 5d). As expected no co-localization between actin patches and the GFP-*Fnk* fluorescence was detectable.

Further studies are needed to reveal the proteins mediating *Fnk* association with the cell membrane and cytoskeletal elements. Preliminary data indicate that microtubules and vimentin, or the PKC-substrates MARCKS are not localized in a manner to suggest codistribution with *Fnk* (data not shown).

Fnk *interacts with the* Ca^{2+}*/integrin-binding protein* Cib

Using the yeast two-hybrid-method and pull down assays we determined an interaction of *Fnk* and *Cib* (Kauselmann *et al*., 1999), a gene previously identified as Ca^{2+}/integrin binding protein (Naik *et al*., 1997). Deletion studies indicated that this interaction depends on the polo-box of *Fnk*. Co-localization studies were performed to gain further support for an interaction in mammalian cells. *Fnk* was expressed in COS cells as a C-terminal fusion of GFP either alone or in combination with a hemagglutinine (HA)-tagged *Cib* protein. Fluorescence staining of the cells was determined after immunological detection of HA-*Cib* using a CY3-labelled secondary antibody by CLSM. Expression of HA-*Cib* alone resulted in a diffuse cytoplasmic and an

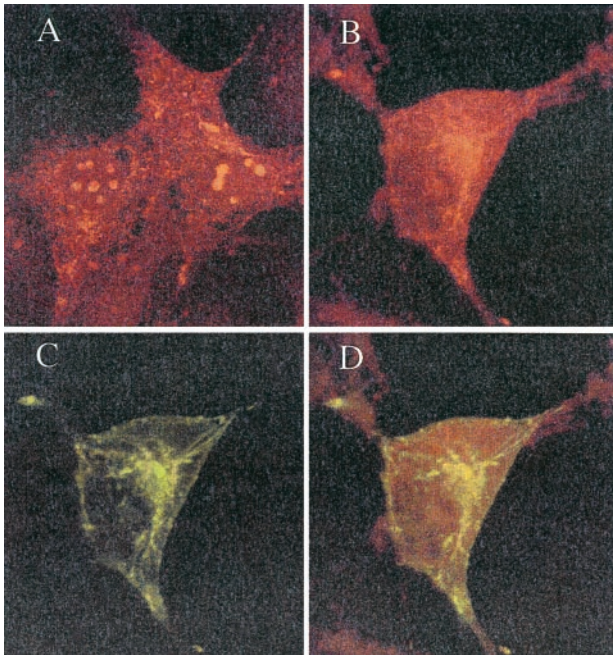

Figure 6 *Fnk* and *Cib* co-localize in COS cells. Cells were transfected with HA-tagged *Cib* alone or with expression plasmids coding for GFP-*Fnk* fusion proteins and HA-tagged *Cib*. Forty hours after transfection cells were fixed and the CY3 as well as the GFP-fluorescence was determined by CLSM. (**a**) Single optical section (by CLSM) taken through a COS cell culture expressing HA-tagged *Cib*. Overview of two *Cib*-positive cells showing cytoplasmic and nuclear staining. Single optical section taken through a COS cell culture expressing GFP-*Fnk* (green) and immunostained for *Cib* (red). (**b**) *Cib*-staining, (**c**) GFP-*Fnk* distribution, (**d**) combination of (**a** and **b**). *Fnk* showed the usual membrane localization and some cytoplasmic granules, whilst *Cib* was mostly found associated to cytoplasmic *Fnk*. Nuclear localization of *Cib* was not observed in cotransfection experiments

additional nuclear staining of COS cells (Figure 6a). In a minor fraction of transfected cells (approximately 1/10) CIB localized exclusively in the nucleus, either distributed diffusely in the karyoplasm or focussed to or surrounding the nucleoli (data not shown). Compared to HA-*Cib* single-transfected cells, coexpression with a *Fnk*-GFP fusion resulted in a redistribution from diffuse to the sites of GFP-fluorescence. However, co-localization was limited predominantly to cytoplasmic *Fnk*, whereas only small amounts of HA-*Cib* were detectable at the cytoplasmic membrane (Figure 6b–d). This observation raised the possibility of a regulated mechanism for *Fnk*/*Cib* binding. Co-localized proteins, which were found in the cytoplasm, do not associate with the nuclear membrane but concentrate in the Golgi-zone. These data provide further evidence for the interaction of *Cib* and the *polo*-related kinase *Fnk* observed in the two-hybrid-system.

To test the interaction between *Fnk* and *Cib in vivo* in more detail, both proteins were transiently expressed in 293T cells. Lysates were subjected to immunoprecipitation with HA antibodies or mouse IgG (negative control). For the following Western blot analysis GFP antibodies were used. The same membrane was stripped and blotted again with HA antibodies. As shown in Figure 7 GFP-*Fnk* could be co-immunoprecipitated with HA antibodies from GFP-Fnk/HA-Cib expressing cells, revealing that *Fnk* and *Cib* associate also in mammalian cells.

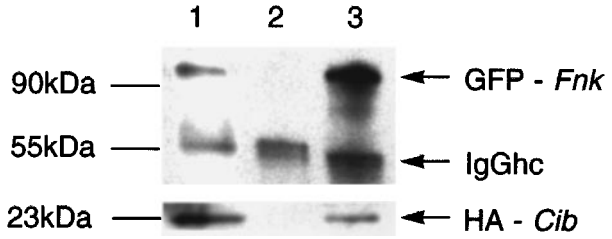

Figure 7 Association of *Fnk* and *Cib* in mammalian cells. Upper panel: Lysates from 293T cells expressing GFP-*Fnk* and HA-*Cib* were subjected to Western blotting using GFP antibodies (lane 1). The additional band at 55 kDa represents a degraded form of GFP-*Fnk*. Lysates from 293T cells expressing GFP-*Fnk* and HA-*Cib* were immunoprecipitated with control mouse IgG (lane 2) or HA antibodies (lane 3). Immunoprecipitates were probed on a Western blot with GFP antiserum. A band of approximately 55 kDa corresponds to the heavy chain of IgG antibodies. Lower panel: Subsequently, the membrane was stripped and reprobed with monoclonal HA antibodies

Taken together, several lines of evidence link the process of cellular adhesion to the expression of the *polo*-related kinase *Fnk*, which may itself also play a role in *Cib*/integrin-mediated signal transduction pathways.

Discussion

In this study we identified a member of the family of serine/threonine kinases by using a PCR-based search for *Plk*-related genes. This kinase gene appears to be the human counterpart of murine *Fnk* due to its close homology of 91% (Donohue *et al*., 1995). Together with *Plk* and *Snk*, human Fnk belongs to a family of protein kinase genes that are closely related to Drosophila melanogaster *polo* and Saccharomyces cerevisiae *CDC5* (Sunkel and Glover 1988; Llamazares *et al*., 1991). Li *et al*. (1996) isolated a human gene, called *Prk* (proliferation related kinase), which displays an amino acid homology of 91% compared to mouse *Fnk*. Sequence alignment of *Fnk* and *Prk* revealed that both cDNAs represent the same human gene. However, *Fnk* exhibits an elongated aminoterminus of 39 aa. In contrast to *Prk*, we found that the entire open reading frame of *Fnk* has the same length as its murine counterpart. The high thermodynamic stability of the 5′ region of the *Fnk*/*Prk* cDNA could be a reason for a premature termination of a cDNA synthesis resulting in an incomplete aminoterminal portion of *Prk*.

The expression of *Prk* was shown to be restricted to certain adult tissues such as lung and placenta. Furthermore, in the analysis of lung tumours and surrounding normal tissues a down-regulation of *Prk* mRNA expression was observed (Li *et al*., 1996). In contrast, we detected an ubiquitous expression of *Fnk* in adult tissues. In our experiments we observed only in certain lung tumours a moderate downregulation of *Fnk* transcript levels compared to surrounding normal tissues from the same patient (Figure 1). Despite the homology to *Plk*, the mRNA expression of *Fnk* differs markedly: The analyses of lung tumours, resting and mitogen stimulated lymphocytes as well as serum-stimulated A431 cells demonstrated that *Fnk* mRNA expression is not associated to the mitotic index.

Previous observations by Donohue *et al*. (1995) demonstrated that murine *Fnk* is an immediate early gene by virtue of the kinetics of *Fnk* expression after FGF-1 stimulation of NIH3T3 cells. *Fnk* transcripts of 2.4 kb were rapidly and transiently expressed following FGF-1 stimulation. In NIH3T3 cells *Fnk* expression, which was induced within 0.5 h after serum addition, declined rapidly and returned to basal levels 8 h after serum addition. In addition, phorbol ester PMA and serum triggered *Fnk* mRNA levels with kinetics similar to those observed after FGF-1 treatment. In contrast, human macrophages of several donors showed a strong expression of *Fnk* mRNA which started 2 h after stimulation and persisted at least 7 days (Figure 2a).

We cultivated primary monocytes as well as cell lines of hematopoietic origin (HL60, U937 and THP1) under the influence of serum and vitamin D_3 to evaluate the correlation between the stage of cellular differentiation and *Fnk* expression (Figure 2). However, cell maturation induced by serum or vitamin D_3 did not increase transcript levels significantly.

Our studies suggest that the adhesion of monocytes rather than the cellular maturation is sufficient for the induction of *Fnk*. Adhesion of monocytes plays a key role in several physiological processes such as diapedesis of monocytes at sites of inflammation and monocyte migration within the extracellular space (Hynes, 1992; Juliano and Haskill, 1993; Hughes and Pfaff, 1998). Despite the importance of monocyte adhesion for many malignant processes the biochemical details of signal transduction by adhesion receptors are poorly understood. The interaction of monocytes with endothelium is believed to be mediated by three types of adhesion molecules: the integrins, the selectins and the Ig superfamily members (Hynes, 1992; Juliano and Haskill, 1993). We treated monocytes with MAb against the α5-subunit of integrins to influence adhesion mediated signalling by these receptors. However, integrin interaction with specific monoclonal antibodies did not alter *Fnk* expression (data not shown). This observation proposes that signalling pathways resulting in *Fnk* mRNA expression are triggered by receptors other than $\alpha_5\beta_1$- or $\alpha_5\beta_3$-integrins. However, a two-hybrid-analysis, co-immunoprecipitation and co-localization studies revealed that the Ca^{2+}/integrin-binding protein *Cib* is an interacting partner of *Fnk* which links this kinase itself to adhesion mediated signal transduction pathways.

Since *Fnk* is located at the cellular membrane but does not contain known membrane binding motifs, an indirect mode of binding had to be considered. One of the most promising candidates was the ubiquitous submembranous actin cortex. If *Fnk* would be targeted to the membrane via the actin cortex, its disruption by cytochalasin D should alter the *Fnk*-distribution. In cells expressing GFP-*Fnk* strongly, the actin cortex seems to become disassembled and the cells gain a spherical morphology. The morphological analysis of the distribution of *Fnk* and f-actin revealed that zones with enhanced *Fnk* presence are deprived of f-actin. Thus, *Fnk* might reduce f-actin stability and as a consequence add to the mobility of monocytes after an initial phase of spreading. In different cell types, for example endothelial cells, the early spreading phase is accompanied by enhanced actin polymerization which immobilizes the cells by the formation of actin bundles (Bereiter-Hahn *et al*., 1990). *Fnk* could counteract this immobilization by rendering the actin cytoskeleton more dynamic.

Our data suggest that *Fnk* influences the architecture of mammalian cells. The activity of *Plk* is also required for functional changes of macromolecular structures such as proper assembly and function of the bipolar mitotic spindle (Lane and Nigg, 1997; Glover *et al*., 1998). *Polo*-related kinases phosphorylate CHO1/MKLP-1, β-tubulin and an 85 kDa microtubule-associated protein (Lee *et al*., 1995; Feng *et al*., 1999; Tavares *et al*., 1996). During the transition from interphase to mitosis, a dramatic reorganization of the microtubule network occurs to form the mitotic spindle. These data indicate that *Plk* interacts with macromolecular structures involved in complex changes of the cellular architecture. Regulation of cell adhesion includes coordinated interactions with the actin cytoskeleton. However, regulation of the cellular morphogenesis requires complex interactions between the adhesion receptors, the cytoskeleton and networks of signalling pathways (Clark and Brugge, 1995). Furthermore, signals generated locally by adhesion can interact with classical signal transduction pathways controlling cellular mRNA expression. This coupling between physical adhesion and intracellular signalling provides a mechanism to adapt cellular function to the local environment. Our data indicate that the expression and the localization of *Fnk* coincide with the major structural reorganization which is required for cellular adherence of macrophages. Interestingly, an inverse pattern of *Snk* regulation was detected in these cells. Due to these results we like to speculate that the closely related members of the family of *polo*-like kinases might be directly involved in the alteration of the cellular morphogenesis.

Materials and methods

Plasmids

The GFP expression vector pEGFPC1 was obtained from Clontech. Recombinant plasmids were constructed by inserting the coding sequences of *Fnk*, *Snk* or *Plk* into pEGFPC1 resulting in GFP fusion proteins (Chalfie *et al*., 1994). An *Eco*RI/*Xho*I fragment of pGEX-*Cib*-HA (influenza virus hemagglutinin epitope) encoding the open reading frame of *Cib* plus 9 aa of HA-tag was cloned into pcDNA3 (Invitrogen).

RNA isolation, Northern blots and PCR

RNA isolation, separation, hybridization, first strand cDNA synthesis and PCR were performed as described previously (Holtrich *et al*., 1991). Primer sequences were as follows: *Plk*-deg2 (WVXKWVDYS-motif): 5′-TTTTGAATTCTGGGTX-ACXAAA/GTGGGTXGAT/CTA-3′; *Plk*-deg3 low (DHTK-XIXC-motif): 5′-TTTTGAATTCCAXAXXATXAXC/TTT-XGTG/ATGA/GT-3′; P12(T+): 5′-GCAGAATTCGTGAA-CTGCGGCCGCA$(dT)_{12}$-3′; P12(T-): 5′-GCAGAATTCGT-GAACTGCGGCCGCA-3′; EB: 5′-CTGAATTCGGATCC-GACTGGTCTGACTCG-3′; EBcom: 5′-CGAGTCAGACC-AGTCGGATCCGAATTCAG-3′; E3: 5′-CTGTTTAGTGA-TGGCACTGTC-3′; *Fnk*-l3: 5′-GTTGATACCCAAAGCC-GAAC-3′; *Fnk*-l7: 5′-CCAACAAGCGGCCTTTGAGG-TAG-3′; Fnk_{ex1}: 5′-CCCCTTGCCCAACAAGCGGCC-3′; Fnk_{ex2}: 5′-CGCGGCGCGGGTCCGTGATG-3′; Snk_{low}: 5′-TTCTTCATTTTGGCTACAGATG-3′.

Labelling of probes

Probes corresponding to aa 285–497 of *Plk*, aa 280–479 of *Fnk* and aa 390–499 of *Snk* were generated using PCR. Radiolabelling of the antisense strand was performed using primers *Plk*-17 low (Holtrich *et al*., 1994), *Fnk*-13 or Snk_{low} in the presence of 150 μCi [γ-^{32}P]dCTP (6000 Ci/mmol; 1 Ci = 37 GBq).

5′ and 3′ elongation by the RACE technique

Specific primers (Fnk_{ex1}, Fnk_{ex2}) from the 5′ region of the *Fnk*-PCR product were utilized for cDNA synthesis. The cDNA was subsequently ligated to a 3′ modified oligonucleotide (EB) and amplified using primer EB(com) and the specific primer *Fnk*-l7 resulting in the extension of 560 bp of upstream sequences. Anchored PCR was performed to complete the missing 3′ portion: a cDNA starting at the poly A-tail was synthesized with P12(T+). Subsequent PCR amplification with the specific primer E3 located at the 3′ end of *Fnk* and primer P12(T−) provided the complete open reading frame.

Cell culture and transient transfections

Peripheral blood mononuclear cells (MNC) were separated by leukapheresis of blood from healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque. Monocytes (MO) were isolated from MNC by countercurrent centrifugal elutriation. MO were >90% pure as determined by morphology and expression of CD14 antigen. Human lymphocytes and macrophages were cultivated as described previously (Briesen *et al*., 1990). Cells were cultured in roller bottles (to prevent adhesion) or in plastic dishes with or without 2% pooled human AB-group serum and antibodies for the indicated time periods. MO were cultivated in the presence of promoters for cellular maturation: Vit. D_3 (Vitamin D_3): 10^{-8} M (1,25-$(OH)_2$-vitamin D_3), LPS (lipopoly-saccharide): 100 ng/ml and IFNγ (interferon gamma): 200 U/ml.

COS-1, 293T (embryonal kidney) and A431 cells (ATCC) were grown at 37°C in DMEM (Bio Whittaker) supplemented with 10% (v/v) heat-inactivated bovine calf serum and a 1 : 100 dilution of a penicillin-streptomycin-solution. The cells were transfected with Lipofectamine (Gibco–BRL) following the manufacturer's recommendations. U937, THP1 and HL60 cells were cultured in RPMI 1640 (Biochrom KG, Berlin) supplemented with 15% foetal calf serum.

Antibodies and chemicals

The antibodies used in blocking experiments were purchased from the following companies: anti-αL-integrin (Coulter-Immunotech, Krefeld) anti-α4-integrin (BIOMOL, Hamburg) and anti-α5-integrin (Calbiochem, Eschborn). Fibronectin and the fibronectin-like engineered protein polymer were from Sigma (Deisenhofen).

Cell lysis, immunoprecipitation and Western blotting

Sub-confluent cells were double transfected with 4 ug of each pGEX-*Cib*-HA and pEGFPC1-*Fnk* using lipofectamine (Gibco–BRL) following the manufacturer's recommendations. Forty-eight hours later cells were harvested and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 10% (vol/vol) glycerol, 1 mM Na_3VO_4, 1 mM DTT) freshly supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 ug/ml aprotinin, pepstatin and leupeptin. Cell lysates were incubated on ice for 1 h with occasional mixing. Subsequently 20 ul of protein A/G plus-agarose beads (Santa Cruz Biotechnology) were added and lysates were precleared by centrifugation for 5 min at 2000 *g*. The supernatant was incubated for 90 min with 2 ug HA antibodies (Clontech) or 2 ug mouse IgG (negative control). Then, protein A/G plus-agarose beads were added and incubated overnight at 4°C. Recovered beads were washed four times, boiled for 3 min in sample buffer and loaded on 12% SDS–PAGE. Precipitated proteins were analysed by Western blotting using GFP antibodies (Clontech) at a dilution of 1 : 100 followed by ECL (Pierce).

Immunofluorescence and microscopy

Cells were washed with PBS and fixed for 15 min at 0°C with either 100% methanol for cell permeabilization, or 4% paraformaldehyde in PBS for cell surface staining. Samples were blocked with 10% calf serum in PBS for 15 min at room temperature. Samples were examined in dual channels using a CLSM (Leica, Wetzlar) and images processed using Imaris software (Bitplane, Zürich).

Cib was visualized by using as first antibody a mouse monoclonal anti-HA tag antibody (BabCo) at a 1 : 500-dilution and a goat anti-mouse CY3 antibody as secondary antibody (Dianova, Hamburg) at a 1 : 100-dilution.

Abbreviations

aa, amino acids; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein.

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