Elevated ERK-MAP kinase activity protects the FOS family member FRA-1 against proteasomal degradation in colon carcinoma cells

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Summary
The AP-1 (activator protein-1) complex, which consists of proteins of the Fos and Jun families, is thought to play an important role in the balance between cell proliferation and apoptosis, the response to genotoxic stress and cell transformation. In cells containing oncogenic Ras, the major components of AP-1 are Fra-1 and c-Jun. signalling from Ras to AP-1 is through the Raf/MEK/mitogen-activated protein (MAP) kinase kinase/ERK (extracellular signal-regulated kinase) MAP kinase pathway as sustained activation of Raf1 or Mek1 modifies AP-1 composition and activity. To analyse the potential link between the ERK-MAPK pathway and AP-1 in colon cancer, in which RAS and BRAF mutations are frequent, we have studied the regulation of AP-1 in colon carcinoma cell lines. We show that c-JUN and FRA-1 expression is dependent on ERK activity and that different thresholds of ERK activity control the expression of FRA-1. A basal activity is required to induce transcription of the FRA-1 gene, but additional higher levels of activity stabilize FRA-1 against proteosome-dependent degradation. These results provide a clear-cut example that the magnitude of ERK signalling affects the cellular response. Although we find no contribution of FRA-1 towards cell proliferation of adherent tumour cells, the high levels of FRA-1 in cells where elevated ERK activity leads to protein stabilization provide survival signals for tumour cells removed from the extracellular matrix.

Key words: ERK-MAPK, AP-1, FRA-1, transcription, degradation, anoikis

Introduction
Oncogenic mutations in the RAS genes are present in a significant proportion of malignant human tumours such as colon (50%) and pancreatic cancers (90%) (Bos, 1989). In cell culture, oncogenic Ras proteins confer tumorigenic and metastatic properties to immortalised rodent cells and to human and rodent primary cells, with the contribution of cooperating oncogenes (Hahn et al., 1999; Land et al., 1983). Three classes of Ras effectors are now well established: the Raf family of protein kinases, phosphatidylinositol 3-kinases (PI3Ks) and the Raf guanine nucleotide exchange factors (RalGEFs) (Downward, 1998; Marshall, 1996; Wolthuis and Bos, 1999). The Raf kinases phosphorylate the dual specific kinases Mek1 [mitogen-activated protein (MAP) kinase kinase 1] and Mek2, which in turn phosphorylate and activate the MAP kinases ERK1 and ERK2 (extracellular signal-regulated kinases). The importance of signalling through Raf in colon cancer is emphasised by the observation that 10-15% of colorectal tumours contain activating oncogenic mutations in BRAF (v-raf murine sarcoma viral oncogene homolog B1) rather than oncogenic mutations in KRAS (Kirsten rat sarcoma viral oncogene homolog) (Davies et al., 2002). The ERK-MAPK signalling pathway activated by Raf appears to be of crucial importance given that its inhibition abolishes the growth of colon tumours in mice (Sebolt-Leopold et al., 1999).

Once activated, the ERK kinases translocate to the nucleus to modulate gene expression through the activation of transcription factors such as those of the Ets domain (Elk-1, Sap-1a) and STAT (signal transducer and activator of transcription) families (Hill and Treisman, 1995). The AP-1 (activator protein-1) family of transcription factors are known to be secondary transcriptional targets of ERK signalling (Balmanno and Cook, 1999; Hill et al., 1993). AP-1 consists of dimers composed of transcription factors of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families (Angel and Karin, 1991). AP-1 has been shown to be a major mediator of transformation by Ras in fibroblasts. In NIH3T3 cells, transformation by Ras induces constitutive expression of c-Jun and Fra-1, which by themselves can promote a transformed phenotype (Mechta et al., 1997). The signalling pathway from Ras to AP-1 appears to be through the Raf-ERK MAPK cascade, as signalling through Raf (Cook et al., 1999) and Mek1 (Treinies et al., 1999) modifies AP-1 activity and composition. The importance of c-Jun activity for Ras-dependent transformation is illustrated in several studies in which c-Jun inhibition has been shown to revert the transformed phenotype induced by Ras in fibroblasts (Johnson et al., 1996; Lloyd et al., 1991; Suzuki et al., 1994). All the Fos family members display oncogenic activities (Bergers et al., 1995; Miller et al., 1984; Nishina et al., 1990; Schuermann et al., 1991); however, the requirement for c-Fos in Ras-mediated transformation is not clear (Hu et al., 1994; Ledwith et al., 1990; Saez et al., 1995). Significantly, Fra-1 rather than c-Fos is the predominant Fos family protein that contributes to
AP-1 activity in Ras- and Mek1-transformed fibroblasts (Mechta et al., 1997; Treinies et al., 1999).

We now show that the c-JUN and FRA-1 components of AP-1, but not JUNB, FRA-2 and c-FOS, are downstream targets of the ERK-MAPK pathway in human colon carcinoma cell lines containing activated KRAS oncogenes. Interestingly, our data indicate that different threshold levels of MAPK activity control transcription and stability of FRA-1. Basal ERK-MAPK activity induces transcription of the FRA-1 gene, whereas higher levels of activity antagonize the proteasome-dependent degradation of the FRA-1 protein and thereby lead to elevated protein levels without affecting the level of FRA-1 mRNA. We also show that elevated FRA-1 levels provide a survival signal for colon carcinoma cells in the absence of attachment to extracellular matrix.

Materials and Methods

Cell culture
BE, HCT-116 and LS174T human colon carcinoma cell lines were grown routinely in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS). For inhibition of MEK1 kinase activity, cells were grown for 36 hours in low serum condition (1% FCS), then 10 μM of the synthetic MEK inhibitor U0126 (Promega #V1121), or 0.1% ethanol in the control experiments, were added. Alternatively, 1 μM of another MEK-specific inhibitor PD184352 was used (gift from P. Cohen, University of Dundee, UK). The HCT-116-RAF:ER cell line was a gift from E. Sahai (Institute of Cancer Research, London, UK) (details available on request). For RAF:ER activation, cells were grown for 36 hours in DMEM supplemented with 1% FCS, then 1 μM 4-hydroxytamoxifen (Gibco # H7904), or 0.1% ethanol in the control experiments, was added. For assessment of the proteasome degradation of FRA-1 protein, cells were treated with 1 μM of the proteasome inhibitor lactacystin (BIOMOL #P5618a) for 16 hours.

Northern blotting
Total RNA was extracted using Rneasy Mini kit (Qiagen) and northern blot analysis was performed using conventional methods. Radioactive probes were coding sequences of the human genes encoding FOSL1 (FOS-like antigen 1) (IMAGE clone 526652), c-JUN (IMAGE clone 322459) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blotting and antibodies
For analysis of ERK, phospho-ERKs and RAF1 protein accumulation, total cell extracts were recovered. For analysis of AP-1 protein accumulation, cell pellets were resuspended in 1 volume of 20 mM Hepes pH 7.9, 600 mM KCl, 0.2 mM EDTA, 1 mM DTT (dithiothreitol), 1 mM vanadate, 1 mM PMSF, 1% aprotinin. After a 30 minute incubation at 0°C, the cell lysate was centrifuged and the supernatant was added to 1 volume of 20 mM Hepes pH 7.9, 0.2 mM EDTA, 1 mM DTT, 40% glycerol, 1 mM vanadate, 1 mM PMSF, 1% aprotinin. Western blot analysis was performed using conventional methods. Antibodies were purchased as follows: anti-phosphorylated forms of ERK1 and ERK2 from Sigma (M8159), anti-ERK2 from TransLabs (E16220), anti-oestrogen receptor alpha from Santa Cruz (sc-543), anti-FRA-1 from Santa Cruz (sc-605), anti-c-JUN from Santa Cruz (sc-7481), anti-JUNB was from Santa Cruz (sc-46), anti-FRA-2 from Santa Cruz (sc-171) and anti-c-FOS from Santa Cruz (sc-052).

RNAi
Oligonucleotides were purchased from Dharmaco (Lafayette, CO). The siRNA sequences targeting human FRA-1 were sense: CACCAUGAGUCCAGUCAGdTdT and antisense: CUGACUGCCACUAGGGUdTdT. These oligonucleotides were chosen to be specific for FRA-1 to avoid silencing of other FOS family members. The scrambled oligonucleotides correspond to the inverse sequences. siRNAs were transfected using the oligofectamine reagent (Invitrogen), following the manufacturer’s recommendations. Cells were used for 48 hours after transfection for western blot analysis of FRA-1 expression and for all biological analyses.

Results and Discussion
Expression of c-JUN and FRA-1 is dependent on the ERK-MAPK pathway in colon carcinoma cells
Because the ERK-MAPK pathway may be constitutively activated by oncogenic mutations in RAS or BRAF, we sought to analyse the contribution of this pathway to the expression of AP-1 in colon carcinoma cell lines in which mutation of these genes occurs. We used two cell lines – HCT-116, which contains an oncogenic KRASG13D mutation, and BE, which contains an oncogenic KRASG13D mutation as well as the BRAF oncogenic mutation, G463V (Davies et al., 2002). We used a phospho-specific ERK antibody to show that ERK-MAPK activity in these two cell lines was constitutively activated even after prolonged growth in low serum conditions. In BE cells, which have both KRAS and BRAF mutations, the MAPK activity is approximately fivefold stronger than in HCT-116, which only contains a KRAS mutation, although the levels of ERK protein expression are identical (Fig. 1A). To assess the role of ERK-MAPK activity on AP-1 expression we used the synthetic MEK1/2 inhibitor U0126 to inhibit ERK phosphorylation. We found that U0126 reduced ERK phosphorylation by more than 90% in both cell lines, as soon as two hours after addition of the MEK inhibitor, and this inhibition persisted for more than 24 hours (Fig. 1A).

We next analysed the expression of the different AP-1 components in HCT-116 and BE cells. We performed these experiments in conditions deprived of serum so that serum growth factors did not obscure the contribution of the oncogenes to MAPK activity. We found that c-FOS, JUNB and FRA-2 proteins were expressed at similar levels in the two cell lines. By contrast, c-JUN and FRA-1 protein expression was threefold to fivefold higher in BE cells compared with HCT-116 cells, which correlated with the difference in MAPK activity (Fig. 1B,C). In agreement, in LS174T colon carcinoma cells, which have a very low MAPK activity (five times less than HCT-116), very low FRA-1 or c-JUN expression, or none at all, was detected (Fig. 1C). In both BE and HCT-116, FRA-1 expression and for all biological analyses.

Cell assays
To assess the role of FRA-1 in the survival of the tumour cells in suspension, control BE, HCT-116 and HCT-116-RAF:ER cells, as well as FRA-1 (or scrambled) silenced cells, were plated on poly-L-Hema (Sigma P-3932)-coated plates for 24 hours and the cell-cycle distribution was determined by FACS analysis. Cells in the sub-G1 phase of the cell cycle were considered dead. In addition, the contribution of the ERK-MAPK pathway was evaluated by adding 1 μM of PD184252 (or DMSO alone) to BE and HCT-116 cells or 1μM hydroxy-tamoxifen (4-OHT) to HCT-116-RAF:ER cells in suspension. Alternatively, the cell-cycle distribution of adherent control cells, FRA-1 silenced cells and cells treated with PD184352 for 24 hours was analysed.

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1 was present as three distinct bands, which probably corresponded to different phosphorylation states (Gruda et al., 1994). JUND and FOSB proteins could not be detected in these carcinoma cell lines (data not shown). These results suggested that expression of c-JUN and FRA-1 but not the other AP-1 components might be dependent on the ERK-MAPK activity. We then analysed expression of AP-1 after inhibition of ERK phosphorylation (Fig. 1B). We found that expression of c-FOS, JUNB and FRA-2 was not significantly affected by ERK inhibition. By contrast, FRA-1 protein levels were dramatically reduced in both cell lines. c-JUN expression was also reduced by ERK inhibition in BE cells. In HCT-116, however, c-JUN expression, which was very low, was not significantly downregulated any further. Altogether, these results indicate that the expression of FRA-1 and c-JUN protein is dependent on the ERK-MAPK pathway in BE and HCT-116 carcinoma cell lines and that accumulation of these two proteins is directly correlated with the level of ERK activity. In RAS-transformed fibroblasts, c-JUN and FRA-1 are also the predominant AP-1 members (Mechta et al., 1997), therefore a common theme in RAS transformation seems to be the overexpression of c-JUN and FRA-1, which suggests a crucial role for these two transcription factors in tumorigenesis. Recent reports indicate that FRA-1 can be phosphorylated by the ERK-MAPK pathway (Hurd et al., 2002; Young et al., 2002). We found that in HCT-116, the three bands corresponding to three phosphorylation states were equally distributed, whereas in BE cells with higher ERK activity, the two slowest migrating bands likely to correspond to phosphorylated forms were predominant (Fig. 1B,C). However, after U0126 treatment the overall pattern of phosphorylated proteins remained the same. This suggests that either FRA-1 is phosphorylated by other kinases or that if it is phosphorylated by the ERK-MAPK pathway, the dephosphorylation process is also inactivated after the inhibition of MEK.

We next investigated whether the regulation of c-JUN and FRA-1 expression occurred at the transcriptional level. We therefore analysed the accumulation of the FRA-1 and c-JUN mRNAs in BE and HCT-116 cells before and after treatment with U0126 (Fig. 1D). We observed that FRA-1 mRNA accumulation was rapidly downregulated after the addition of the MEK inhibitor in both cell lines. However, in contrast to FRA-1 protein, the FRA-1 mRNA was present in almost identical amount in control BE and HCT-116 cells. These results indicate that MAPK regulation of FRA-1 expression occurs at the level of mRNA accumulation, but that another ERK-dependent mechanism may influence the accumulation of the protein in BE cells. In the case of c-JUN, we found that the mRNA was expressed at a significant level only in BE cells, and this was directly correlated with the levels of c-JUN protein. In BE cells, inhibition of the MAPK pathway resulted in downregulation of c-JUN mRNA accumulation, confirming that in those cells regulation of c-JUN expression occurs at the mRNA level. Surprisingly in HCT-116, inhibition of ERK led to an upregulation of c-JUN mRNA accumulation. These results suggest that high levels of MAPK activity (BE)
positively regulates c-JUN transcription, whereas low levels (HCT-116) are negative regulators, and that these differences are responsible at least in part for the different accumulation of c-JUN protein in BE and HCT-116 cells. The absence of c-JUN protein, in spite of mRNA expression after MEK inhibition in HCT-116, suggests that another level of regulation antagonizes its accumulation in these cells.

It has been reported that U0126 also inhibits the MEK5-ERK5 pathway (Kamakura et al., 1999). To rule out the possibility that in our cells the expression of c-JUN and FRA-1 is dependent on the MEK5-ERK5 pathway, we treated the cells with another MEK inhibitor, PD184352, at a concentration (1 μM) known to inhibit the classical ERK-MAPK pathway only (Mody et al., 2001). We found that expression of c-JUN and FRA-1 proteins (Fig. 1E) and mRNAs (data not shown) were efficiently downregulated by 1 μM PD184352 in BE cells. PD184352 had also similar effects to U0126 in HCT-116 cells (data not shown). These data therefore show that it is ERK1/2 rather than ERK5 that contributes to FRA-1 and c-JUN expression.

Altogether, these results indicate that expression of c-JUN and FRA-1 in colon carcinoma cells is dependent on the ERK-MAPK activity, and that this occurs at least in part at the level of mRNA accumulation. This could be achieved either by mRNA stabilization or, more likely, by increased transcription. The promoter of the FRA-1 gene contains numerous homologies with the c-FOS promoter (Tsuchiya et al., 1993), including the serum response element (SRE) responsible for the transactivation by the ternary complex factors (TCFs), which are induced by serum and the ERK-MAPK pathway. Ternary complex factors bound to this element in the FRA-1 promoter might be responsible for the transcription of FRA-1 in HCT-116 and BE colon carcinoma cells, in response to the ERK-MAPK pathway. Alternatively, a recent report suggests that FRA-1 transcription in response to RAS transformation in a rat thyroid cell line is mediated by a positive autoregulation (Casalino et al., 2003). The authors show that FRA-1 transactivates its own gene promoter via binding to an AP-1 regulatory element in the first intron of the gene in a RAS- and ERK-dependent manner. A similar mechanism could take place in colon carcinoma cell lines with activated RAS mutations. Induction of c-JUN transcription by serum, growth factors and insulin is mediated through AP-1 binding sites in the promoter. AP-1 bound to these sites might be responsible for the transcription of c-JUN in BE cells.

Elevated ERK-MAPK activity prevents proteasome-dependent degradation of FRA-1 protein in BE cells

In contrast to c-JUN, the experiments presented above indicated that the difference in FRA-1 protein accumulation between HCT-116 and BE cells could not be explained by a difference in mRNA accumulation. AP-1 components such as c-FOS and c-JUN are known to be degraded by multiple proteolytic mechanisms, including the lysosomes and the ubiquitin-proteasome proteolytic pathway. We tested whether FRA-1 protein was targeted to proteasome-dependent degradation in HCT-116 and BE cells. Addition of the proteasome inhibitor lactacystin had no significant effect on FRA-1 protein accumulation in BE cells, indicating that this protein is not targeted to proteasome degradation in these cells (Fig. 2A). On the contrary, in HCT-116 cells lactacystin induced an increase in FRA-1 protein accumulation to levels almost identical to those in BE cells. These results suggest that FRA-1 protein is degraded via the proteasome pathway in HCT-116 but not in BE, and this contributes to the difference in protein accumulation between the two cell lines.

One explanation for this difference in protein accumulation is that it is due to the higher MAPK activity in BE cells. To test this hypothesis we used a stable variant of HCT-116 overexpressing a fusion protein between RAF1 and the hormone binding domain of the oestrogen receptor (RAF:ER). Addition of 4-hydroxy-tamoxifen (4-OHT) led to a rapid activation of ERK1/2 phosphorylation by threefold, without any modification in the level of ERK proteins (Fig. 2B). We found that on 24 hours of RAF1 induction, FRA-1 protein accumulation was also upregulated by threefold, and the proportion of the slowest migrating band (hyper-
phosphorylated FRA-1) was increased. The increase in protein accumulation was not due to an increased transcription of the FRA-1 gene because the levels of FRA-1 mRNA were not affected by the RAF1 induction (Fig. 2C). This result confirms that the increased accumulation of the FRA-1 protein in BE cells compared with HCT-116 was due to the higher MAPK activity. This also confirms that ERK-MAPK activity participates in the phosphorylation of the FRA-1 protein.

Altogether, our results suggest that different threshold levels of MAPK activity control FRA-1 expression in colon carcinoma cells. The level of ERK-MAPK activity seen in cells like HCT-116 is necessary and sufficient to induce transcription of the FRA-1 gene, but a higher level of ERK-MAPK activity, like in BE cells, is able to prevent the proteasome-dependent degradation of FRA-1 protein and therefore further increase FRA-1 accumulation. Our results suggest that this escape from the proteasome machinery may be the consequence of an increased phosphorylation of the FRA-1 protein by the ERK-MAPK pathway. In agreement with our studies, a very recent study has shown that the stability of the FRA-1 protein is dependent on its phosphorylation status, which is regulated by the ERK-MAPK pathway (Casalino et al., 2003); however, unlike our studies these authors did not make the connection between the stabilisation of FRA-1 and the level of ERK activity. Previous studies have shown that the proteasome-dependent degradation of c-FOS can be inhibited by phosphorylation by the ERK-MAPK pathway, and c-JUN degradation can be inhibited by activation of the JNK (JUN N-terminal kinase) pathway (Musti et al., 1997; Okazaki and Sagata, 1995; Stancovski et al., 1995; Treier et al., 1994). In the case of c-JUN, a high MAPK activity, like in BE cells, is required to induce transcription and protein accumulation.

Altogether, these data therefore argue that in addition to the sustained nature of ERK-MAPK activity resulting from the constitutive activation of RAS or BRAF, the magnitude of the activity is also important in tumours.

FRA-1 protects tumour cells from anoikis
To investigate the role that FRA-1 plays in cellular transformation we silenced the gene expression using a short interfering RNA (siRNA). siRNA almost completely obliterated the expression of FRA-1 (Fig. 3A) in BE and HCT-116 cells and resulted in a major reduction in binding activity to a consensus AP-1 oligonucleotide in gel-shift experiments (data not shown). Surprisingly, in view of previous published work implicating AP-1 activity in the regulation of cell-cycle
progression (for a review, see Shaulian and Karin, 2001). BE or HCT-116 cells in which FRA-1 had been silenced showed no change in cell-cycle profile when cultured in anchorage-dependent, low-serum conditions (Fig. 3B). It is possible that BE and HCT-116 cells have acquired additional genetic lesions that free them from a requirement for AP-1 for proliferation in attached conditions. However, inhibition of activation of the ERK-MAP kinase pathway by PD184352 (or U0126, data not shown) blocked these cells in the G1 phase of the cell cycle, showing that ERK-MAP kinase signalling, but not via FRA-1, is required for the proliferation of these cells. In the search for other properties of tumour cells that might be regulated through FRA-1 we investigated whether FRA-1 might have a role in survival signalling. Normal cells that require attachment to extracellular matrix for survival and proliferation undergo apoptotic death when removed from the matrix – a process known as anoikis (Fisch and Francis, 1994). Many tumour cells have escaped anoikis and can survive and proliferate when separated from the extracellular matrix. Activation of signalling pathways by oncogenes such as Ras can play an important part in this survival signalling (Khwaja et al., 1997). BE and HCT-116 cells are able to proliferate in anchorage-independent conditions but HCT-116 showed a much higher rate of apoptosis under these conditions than BE (subG1 peak = 5% for BE, 19% for HCT-116, Fig. 3B). To investigate whether these differences in survival signalling reflected the differences in FRA-1 levels between the two cell lines, first we showed that silencing FRA-1 increased anoikis almost sixfold in BE cells but led to only a 50% increase in HCT-116 (Fig. 3B). Second, we used the HCT-116-RAF:ER cells to determine whether increasing ERK activity and thereby FRA-1 levels (Fig. 2A) would provide a survival signal. Fig. 3C shows that in the absence of 4-OHT to induce the RAF-ER, the subG1 peak in HCT-116-RAF:ER cells was 14.9%; by contrast, when 4-OHT was added the subG1 peak dropped to 8.8%. This protective effect of activating the RAF-ER is mediated through FRA-1 because silencing FRA-1 through siRNA abrogated the effect of the RAF-ER (Fig. 3C). Previous work has suggested that AP-1 proteins can have pro-apoptotic or anti-apoptotic effects depending on the cellular context (Shaulian and Karin, 2001). Our results suggest that in cells with high levels of ERK signalling FRA-1 acts to provide survival signals. In colorectal carcinoma cells it appears that a key role of AP-1 downstream of the ERK-MAPK pathway is to provide survival signals in the absence of attachment to the extracellular matrix.

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