Molecular Mechanisms of Signal Transduction
J.L. Bos (Ed.)
IOS Press, 2000

Regulation of components of AP-1 transcription factor by early and late Ras signals

A. Pintzas, V. Zoumpourlis and D. Plows
Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48, Vas. Constantinou Avenue, 116 35, Athens, Greece

Abstract. Experiments utilising either tumour cells or over-expression of oncogenes like *Ras* and its potential downstream mediators have yielded a wealth of information over the last decade. Qualitative and quantitative analysis of AP-1 transcription factor has been extensively analysed in response to various oncogenic signals. One basic criticism is that the continued presence of an activated component of cellular signaling renders the study of early *Ras*-mediated signaling impossible. Inducible systems for oncogene expression offer a valuable alternative for detailed analysis of signal transduction pathways. Here, we report the comparative analysis of components of oncogenic pathways between tumour cells and cells that carry inducible oncogenes.

1. AP-1 composition

Transcription factor AP-1 (activator protein 1) plays an important role in cell proliferation, differentiation, apoptosis and malignant transformation. Homo and heterodimeric complexes of Jun, Fos and ATF family members form the DNA binding complex thus regulating transcription.. Homodimers of c-Jun protein and c-Jun/c-Fos heterodimers bind to the AP-1 consensus sequence TGAC/GTCA [1]. C-Jun/ATF-2 heterodimers and ATF-2 homodimers preferably bind to the ATF/CREB consensus sequence TG/TACGTCA [2]. Signal transduction pathways initiated at the cell surface in response to cytokines, growth factors, tumour promoters and overexpression of certain oncogenes [3] finally regulate members of all three families. The *jun* family comprises three members (*c-jun, junB, and junD*) [4-6] and the *fos* family four different genes (*c-fos, fos B, fra-1, fra-2*) [7-9]. Activating transcription factor 2 (ATF-2) is a member of the ATF/CREB bZip family of transcription factors [10]. ATF-2 plays important roles in the early events of signaling pathways, since phosphorylation of this factor by the c-jun-N-terminus-associated kinases increases its trans-activating properties and permits the early activation of the *c-jun* promoter [11]. AP-1 activity is regulated at the level of transcription of *jun* and *fos* genes, by protein-protein interactions and post-translational modifications of Jun and Fos proteins [12-14].

2. Ras signalling and AP-1

The p21Ras proteins are frequently mutated in human tumours [15] and provide a link between receptors at the cell membrane with the cytoplasmic cascade of protein kinases followed by downstream nuclear events, including induction of transcription and DNA synthesis [16]. Mutant Ras proteins have been shown to regulate AP-1 activity at transcriptional and post-translational levels [17-19]. Among the *jun* family genes that encode for transcription factors, only *c-jun* displays full transforming potential in cooperation with activated H-*ras* in primary rat embryo fibroblasts [20]. Phosphorylation of N-terminal c-Jun protein residues Ser-63 and Ser-73 by JNK kinases is necessary for transactivation and oncogene cooperation using *in vitro* transformation assays. AP-1 transcriptional response and the ability to be transformed by activated Ras proteins are markedly impaired in cells carrying a homozygous null mutation of the c-*jun* gene [21]. JunB and JunD can also cooperate with Ras to induce foci, but with less efficiency compared to c-Jun [22-23]. JunD protein has been reported to efficiently suppress transformation by an activated *ras* gene [24]. Investigation of Fos protein levels has shown that Fra-2 protein plays a crucial role in the transformation induced by *H-ras* gene in CEF. In addition, Fra-1 protein induces morphological transformation and increases invasiveness and motility of mouse adenocarcinoma cells [25].

3. Models to study Ras signalling to AP-1

The analysis of multistage carcinogenesis has been facilitated by animal model systems, which have formed the basis for much of our present understanding of the genetic and biological changes involved in the development of solid tumours. In particular, mouse skin has provided the paradigm for studies of multistage carcinogenesis in rodents [26]. Carcinogenesis can be initiated in normal epithelial cells by a single treatment of the skin with a variety of chemical mutagens, which have previously been shown to induce carcinogen -specific mutations in the H-*ras* gene [27]. Mutations in the H-*ras* gene confer a selective advantage upon initiated cells, which then develop into benign tumours after treatment with promoting agents such as 12-O-tetradecanoyl phorbol-13-acetate (TPA).

A series of cell lines representing different stages of mouse skin tumour progression has been created. These include immortalised non-tumourigenic keratinocyte lines, benign papilloma cell lines and squamous carcinoma cell lines, which give rise to well differentiated tumours upon injection into nude mice. Highly anaplastic, invasive spindle cell lines which have been shown to invade *in vitro* and exhibit extremely aggressive tumour growth, including metastasis *in vivo* have been established [28-29]. These cell lines are representative for the development of three distinct stages in mouse carcinogenesis including benign papillomas, locally invasive squamous carcinomas and metastatic spindle carcinomas. All the cell lines have been extensively characterised with regard to the status of a number of oncogenes and tumour suppressor genes, including H-*ras*, *p53*, *cyclin D*, *Rb* and *p16* [29-31; and unpublished results]. These cells therefore allow comparison of the specific changes associated with malignant progression.

We have chosen cell lines that all except the control were obtained from tumours initiated *in vivo* with DMBA, and consequently carry the H-*ras* mutation at codon 61, which is typically induced by this initiating agent [32]. It It has been demonstrated that the mutant H-*ras* gene copy number and expression levels are in line with the level of malignancy of the cell line (S. Frame, R. Crombie and A.B., unpublished). The spindle carcinoma cell line carB also shows relatively high expression of the mutant *ras* allele, but in addition has lost the normal allele [33].

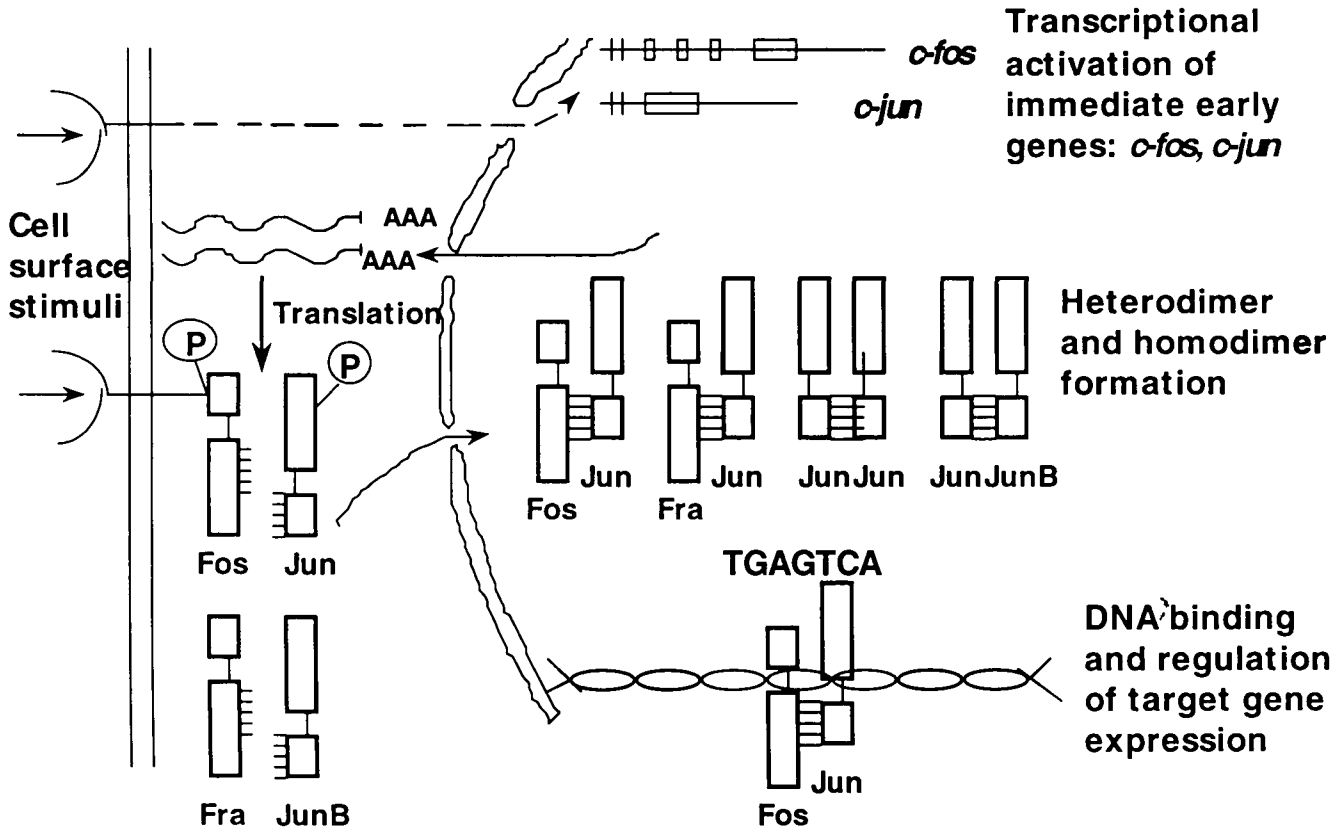

Fig. 1. AP-1 in cell signalling. Schematic representation of the regulation of AP-1 components at multiple levels

We analysed the expression patterns of Jun, Fos and ATF-2 proteins during mouse skin carcinogenesis and we show that phosphorylated forms of c-Jun, Fra-1, Fra-2 and ATF-2 proteins are expressed in higher amounts in the squamous carcinoma cell lines and especially in metastatic cell lines with a spindle morphology. Hyper-phosphorylation of Jun and ATF-2 correlates with high JNK kinase activity in tumor cells. AP-1 DNA binding and trans-activation activity were found elevated in the malignant cell lines, peaking in the fully metastatic cell lines. Our results also suggest, for the first time, that ATF-2 plays an important role in the progression of mouse skin tumours [34].

4. Ras inducible systems

The importance of Ras mutation has been highlighted by the recent report that primary human epithelial and fibroblast cells can be rendered tumourigenic by the ectopic expression of telomerase catalytic subunit (hTERT) in combination with the SV40 T oncogene and the activated (V12G; GTP-bound) mutant allele of Harvey-Ras [35].

Whilst experiments utilising over-expression of Ras or its potential downstream mediators have yielded a wealth of information over the last decade, one basic criticism is that the continued presence of an activated component of cellular signaling renders the study of early Ras-mediated signaling impossible. The employment of the scrape-loading technique whereby milligram amounts of recombinant Ras protein are added to cell cultures partially overcomes this obstacle but the system suffers from the fact that the levels of Ras are not physiologically relevant. We therefore selected an ecdysone inducible gene-expression system [36] in order to investigate early Ras-mediated signaling events in NIH3T3 mouse fibroblasts.

Using this system, we are able to rapidly induce expression of activated (V12G) Ha-Ras in a dose-dependent manner and at physiological levels equivalent to chemically-induced carginogenic epithelial cells derived from BalbC mice. Comparison with NIH3T3 cells over-expressing Ha-Ras (clone R1, a kind gift from M. Yaniv, Institut Pasteur, France) [37] reveals that the peak induction of Ha-Ras is at least 10-fold less than that occurring in the constitutively expressing cells. Upon induction of the activated (V12G) Ha-Ras molecule, NIH3T3 cells display transformation-like characteristics such as morphological change, colony formation in soft agar, and continued growth in the absence of growth factors . Analysis of cellular signaling by Western Blotting reveals a rapid induction of the MAPK pathway (using anti-Phospho ERK1/2 antibody) similar to that reported previously. Interestingly the induced ERK signal is approximately 5-fold stronger than that found in R1 cells that constitutively over-express Ha-Ras and hence are continuously signaling through the MAPK cascade, suggesting a de-sensitisation event has occurred in these cells. Analysis of other signal transduction pathways reveals a delayed but transient induction of the SAPK pathway (anti-Phospho JNK antibody). No activation of the p38 MAPK pathway has been observed. Activation of both pathways was functionally confirmed by kinase assay utilising GST-ELK or GST-c-Jun as the target substrates for ERK and JNK respectively. In addition, a corresponding dose-dependent increase in AP-1 DNA binding activity was observed.. Our results confirm that MAPK is rapidly and constitutively activated by the presence of activated Ras, In addition the data suggests that SAPK is indirectly activated by Ras and implies that activation of SAPK is tightly regulated due to the transient nature of the signal. It is not known whether continuous exposure to Ha-Ras signaling will eventual lead to a change in the composition of the AP-1 complex.

	Systems	
	Cells bearing Inducible Ras	Ras transformed cells
(protein levels as compared with immortalised cells)		
Ras	Increase	Very high
p-Erk	Transient Increase	High
p-JNK	Increase	Increase
p-Jun	Increase	Increase
c-Fos	Increase	Low

Fig. 2. Comparative analysis of AP-1 components between tumour cells bearing Ras mutations and cells containing inducible Ras oncogene.

5. Concluding remarks

In conclusion, these data highlight the differences in detected signal transduction when comparing early to late exposure to Ras, and suggest that the Ecdysone inducible expression of Ha-Ras is a more relevant model in which to investigate potential blockade of Ras-mediated transformation, and in addition might lead to the identification of novel targets for therapeutic intervention.

References

[1] Angel P, Baumann I , Stein B, Delius H, Rahmsdorf HJ and Herrlich P. (1987). *Mol. Cell. Biol* , **7**, 2256-2266.
[2] Benbrook DM. and Jones NC. (1990). Oncogene, 5, 295-302.
[3] Karin M, Liu Zg and Zandi E. (1997). *Curr Opin Cell Biol.*, **9**, 240-246.
[4] Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK and Tjian R (1987). *Science*, **238**, 1368-1392.
[5] Hirai SI, Ryseck RP, Mechta F, Bravo R, and Yaniv M. (1989). *EMBO J.*, **8**, 1433-1439.
[6] Ryder K, Lanahan A, Perez AE and Nathans D. (1989). *Proc. Natl. Acad. Sci.*, **86**, 1500-1503.
[7] Cohen DR and Curran T. (1988). *Mol. Cell. Biol.*, **8**, 2063-2067.
[8] Nishina H, Sato H, Suzuki T, Sato M and Iba H. (1990). *Proc. Natl. Acad. Sci.*, **87**, 3619-3621.
[9] Zerial R, Toshi L, Ryseck RP, Schuerman M, Mueller R and Bravo R. (1989). *EMBO J.*, **8**, 805-813.
[10] Maekawa T, Sakura H, Kanei-Ishii C, Suda T, Yoshimura T, Fujisawa J, Yoshida M and Ishii S. (1989). *EMBO J.*, **8**, 2023-2028.
[11] Van Dam H, Huguier S, Kooistra K, Baguet J, Vial E, Van der Eb A, Herrlich P, Angel P and Castellazi M. (1998). *Genes and Dev.*, **12**, 1227-1239.
[12] Pulverer B, Kyriakis J, Avruch J, Nikolakaki E and Woodget J. (1991). *Nature*, **353**, 670-674.
[13] Gruda M, Kovary K, Metz R and Bravo R. (1994). *Oncogene*, **9**, 2537-2547.
[14] Oehler T, Pintzas A, Stumm S, Darling A, Gillespie D and Angel P. (1993). *Oncogene*, **8**, 1141-1147.
[15] Bos, J.L. (1989). *Cancer Res.***49**, 4682-4689.
[16] Burgering, B.M.T. and Bos J.L. (1995). *TIBS* **20**, 18-22
[17] Smeal T, Binetruy B, Mercola D, Birrer M and Karin M. (1991). *Nature*, **354**, 494-496.
[18] Binetruy B, Smeal T and Karin M. (1991). *Nature*, **351**, 122-127.
[19] Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M and Davis R (1994). *Cell*, **76**, 1025-1037.
[20] Schutte J, Minna J and Birrer M. (1989a). *Proc. Natl. Acad. Sci.*, **86**, 2257-2261.
[21] Johnson R, Spiegelman B, Hanahan D and Wisdom R. (1996). *Mol. Cell. Biol.* **16**, 4504-4511
[22] Schutte J, Viallet M, Nau S, Segal S, Fedorko J and Minna. J. (1989b). *Cell*, **59**, 987-997.
[23] Vandel L, Montreau N, Vial E, Pfarr C, Binetruy B and Castellazzi M. (1996). *Mol. Cell. Biol.*, **16**, 1881-1888.
[24] Pfarr CM, Mechta F, Spyrou G, Lallemand D, Carillo S and Yaniv M. (1994). *Cell*, **76**, 747-760.
[25] Kustikova O, Kramerov D, Grigorian M, Berezin V, Boch A, Lucanidin E and Tulchinsky E. (1998). *Mol. Cell Biol.*, **18**, 7095-7105.
[26] Yuspa SH and Poirier MC. (1988). *Adv. Cancer Res* , **50**. 25-70.
[27] Balmain A and Brown K. (1988). *Adv. Cancer Res* , **51**, 25-70.
[28] Buchmann A, Ruggeri B, Klein-Szanto AJP and Balmain A. (1991). *Cancer Res.* **51**, 4097-4101.
[29] Quintanilla M, Haddow S, Jonas D, Jaffe D, Bowden GT and Balmain A. (1991). *Carcinogenesis*, **12**, 1875-1881.
[30] Burns PA, Kemp CJ, Gannon JV, Lane DP, Bremner R and Balmain A. (1991). *Oncogene*, **6**, 2363-2369.
[31] Linardopoulos S, Street A, Quelle DE, Parry D, Peters G, Sherr CJ and Balmain A. (1995) *Cancer Res.* 55, 5168-5172.
[32] Quintanilla M, Brown K, Ramsden M and Balmain A. (1986). *Nature*, **322**, 78-80.
[33] Bremner R and Balmain A. (1990). *Cell* **61**, 407-417.
[34] Zoumpourlis V., Papassava, P., Linardopoulos, S., Gillespie, D., Balmain, A. and Pintzas, A. Submitted
[35] Hahn et al. (1999) *Nature*, **400**, 464
[36] No et al. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 3346-3351
[37] Mechta F, Lallemand D, Pfarr C and Yaniv M. (1997). *Oncogene*, **14**, 837-847