

A novel *in vivo* assay for the analysis of protein–protein interaction

Marlene Maroun and Ami Aronheim*

Department of Molecular Genetics, Rappaport Family Institute for Research in the Medical Sciences and the B. Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, PO Box 9649, Bat-Galim, Haifa 31096, Israel

Received March 24, 1999; Revised and Accepted May 18, 1999

ABSTRACT

The Ras Recruitment System (RRS) is a method for identification and isolation of protein–protein interaction. The method is based on translocation of cytoplasmic mammalian Ras protein to the inner leaflet of the plasma membrane through protein–protein interaction. The system is studied in a temperature-sensitive yeast strain where the yeast Ras guanyl nucleotide exchange factor is inactive at 36°C. Protein–protein interaction results in cell growth at the restrictive temperature. We developed a gene reporter assay for the analysis of protein–protein interaction in mammalian cells. Ras activation in mammalian cells induces the mitogen-activated kinase cascade (MAPK), which can be monitored using Ras-dependent reporter genes. This greatly extends the usefulness of the system and provides a novel assay for protein–protein interaction in mammalian cells.

INTRODUCTION

Much progress has been made during the last decade in isolation and identification of novel genes and understanding their functions. It is well established that all gene products exert their action through multiple protein–protein interactions. In order to identify the interaction network array of a protein of interest, numerous procedures have been developed. The most powerful genetic method available today is the well-known two hybrid system, originally described by Fields and Song (1). The two hybrid system, which is based on reconstitution of a functional transcription factor through protein–protein interaction, is monitored simply by using reporter gene assays in yeast. The transcription of the reporters is designed under the control of the appropriate DNA target sites of the corresponding transcription factor. The reporter genes that are commonly used are either/or both nutrient markers such as the HIS3 gene, providing the selection of positive interaction on a medium lacking histidine, and/or a reporter gene with a measurable enzymatic activity, such as the β-galactosidase gene, providing blue color selection (2). The use of reporters facilitates the quantification of protein–protein interaction and permits the comparison between different protein pairs and/or mapping of functional interaction domains using either mutations and deletion analysis of known protein interaction. In general, the strength of interaction predicted by the two hybrid system correlates to some degree with affinity data obtained using *in vitro* measurements (3). The two hybrid system, with its constant improvements during the last decade, provides a very powerful method for the analysis of protein–protein interaction. Nevertheless, it exhibits several limitations and problems (4–10).

Recently, two novel protein–protein interaction assays were developed which complement and overcome some of the problems and limitations of the two hybrid system (11,12). These assays are based on a completely different readout to monitor a successful protein–protein interaction in yeast. It is based on translocation of the active molecules, hSos (11) or Ras (12) to their site of action at the inner leaflet of the plasma membrane. Translocation is achieved through protein–protein interaction. Unlike the two hybrid system, this assay is monitored by testing cell viability of an otherwise temperature-sensitive CDC25-2 yeast strain (13) at the restrictive temperature. The protein recruitment systems were proven to be useful for the identification and isolation of known and novel protein interactions (11,12,14,15). Since the detection of cell growth is mainly qualitative and cannot be quantitated easily, this assay is limited in its ability to compare interactions between different protein pairs. Therefore, the addition of reporters to the protein recruitment system would greatly improve the reliability of the assay. In addition, protein–protein interactions that are identified in yeast through library screening for proteins of mammalian origin do not necessarily represent a bone fide interaction when tested in mammalian cells (15,16). Indeed, modified two hybrid assays were developed to detect protein–protein interaction directly in mammalian cells. Thus, this assay better mimics the situation which occurs *in vivo* (17–19). In addition, the use of a mammalian system for proteins of mammalian origin is preferable for a reverse two hybrid approach (20–22). Using such an approach, protein pairs that are known to result in abnormal function in the cell leading to disease or to uncontrolled cell growth are screened for either proteins or drugs that are able to inhibit and compete for their association. The drug screening approach directly in mammalian cells is preferable, since it tests toxicity and permeability of the drug directly within the target cells.

The following paper describes the development of a novel protein–protein interaction assay in mammalian cells, designated

*To whom correspondence should be addressed. Tel: +972 4 8295226; Fax: +972 4 8295225; Email: aronheim@tx.technion.ac.il

the mammalian Ras Recruitment System (mRRS). This assay is based on the recruitment of Ras to the plasma membrane through protein–protein interaction. Ras activation initiates the activation of the MAPK cascade that can be monitored through the use of reporter genes designed under the control of Ras responsive elements. Thus, this assay can be used for quantification and verification of protein–protein interaction identified in yeast, directly in mammalian cells.

MATERIALS AND METHODS

Plasmid construction

The expression plasmids encoding for different fusion proteins (described in 12) were transfered to pcDNA3.1 expression vector (Invitrogen Inc.).

Transfection and luciferase analysis

Cell culture and transfection were performed by conventional calcium phosphate percipitation method. Luciferase activity was performed using the luciferase assay system (Promega Inc.), measured by TD-20/20 luminometer (Turner Designs Ltd). The results represent an average of three to four independent experiments.

RESULTS

Ras activation in mammalian cells results in activation of the MAPK cascade leading to potentiation of transcription of multiple transcription factors through phosphorylation of their activation domain. Several transcription factors were found to be activated in a Ras-dependent manner, for example, proteins from the ETS family such as ELK1/TCF (23), c-Myc (24), glucocorticoid receptor (25) and Tal1 (26). Therefore, reporter plasmids designed under the control of DNA elements corresponding to these transcription factors, known in general as 'Ras responsive elements', are actively transcribed as a result of Ras activation, and are therefore commonly used to measure Ras activity (Fig. 1A). We used reporter plasmids encoding for the chloramphenicol acetyl transferase (CAT) or the firefly luciferase gene product under the control of DNA elements derived from the polyoma virus enhancer (27) or multiple AP-1 sites (28,29), respectively. These reporters were shown to be activated in a Ras-dependent manner. In mammalian cells, cytoplasmic Ras is inactive (30) and is unable to activate the MAPK cascade. This is due to the fact that Ras exerts its downstream effect through the recruitment of Raf1 to the plasma membrane, which is essential for activation of Raf kinase and the MAPK cascade (31). Therefore, overexpression of mammalian cytoplasmic Ras fused to a protein of interest ('the bait') is not expected to activate Ras-responsive reporters (Fig. 1A). On the other hand, translocation of the bait to the inner leaflet of the plasma membrane is expected to result in activation of the Raf kinase and activation of the downstream MAPK cascade, which can be monitored using the different Ras-responsive reporters (Fig. 1B). Translocation of the bait to the plasma membrane could be achieved via interaction of the bait protein with a protein partner ('the prey') fused to membrane localization signals, such as myristoylation. Whereas in yeast, this interaction results in cell growth at the restrictive temperature, in mammalian cells, protein–protein interaction can be monitored using the Ras responsive reporters. In order to test this assay, we transferred the cDNAs of different 'bait' and their corresponding 'prey' protein hybrids to mammalian expression vectors (pcDNA Invitrogen) designed under the control of the cytomegalovirus enhancer and promoter (CMV). Plasmids were cotransfected into HEK-293 cells (human embryonic kidney cell line) using the calcium phosphate precipitation method. Forty hours following transfection, cells were harvested and luciferase and CAT activities were determined. The following protein–protein interactions were tested using the polyoma enhancer element linked to the CAT gene. (i) The interaction between phosphatidylinositol 3-kinase (PI3K) subunits: p110 and p85 (32), as previously described (11,12) (Fig. 2, left panel). (ii) The interaction between Grb2 and hSos (12) (Fig. 2, right panel).

In both cases, neither the bait nor prey plasmid alone results in a significant increase in the transcription levels of the reporter. However, when the corresponding prey and bait plasmids are cotransfected together, a significant synergism is observed (Fig. 2). No increase in activity was observed when M-Grb2 or M-p85 was used with a non-relevant bait expression plasmid such as p110-Ras or 3'Sos-Ras, respectively (data not shown). This indicates that activation of the Ras signaling pathway can be achieved by recruitment of cytoplasmic activated Ras protein to the plasma membrane via protein–protein interaction.

The ability to detect interaction between Pak65 regulatory domain (33) with Chp, a recently isolated small GTPase from the Rho family (14) was tested using multiple AP-1 DNA elements linked to the luciferase gene. Consistently, cotransfection of the Pak65-bait together with the myristoylated Chp expression plasmids results in a significant increase in the reporter activity compared to the activity obtained with Ras–Pak alone (Fig. 3).

DISCUSSION

This paper strongly suggests that the RRS originally developed in a yeast host strain could be transferred to a mammalian-based system. It is clear that protein pairs that strongly affect the MAPK cascade or exhibit strong effects on transcription of the corresponding reporters could not be used in this assay. For example, we failed to detect the interaction between the DNA binding domains of c-Jun and c-Fos in this *in vivo* set-up. This is mainly due to the fact that expression of the DNA binding domains of these transcription factors lacking their transcriptional activation domains act as dominant negative inhibitors for the endogenous AP-1 proteins (34). Therefore, the use of the mRRS would not be suitable for the isolation of novel protein interaction through a library screening approach. Nevertheless, the development of a mRRS significantly improves the usefulness of the yeast RRS. First, the protein–protein interaction initially identified in yeast can be verified directly in mammalian cells. Second, the use of reporters provides a quantification dimension for the strength of interaction and, therefore, the interaction between different mutants or protein partners can be readily compared. Third, once a protein–protein is detected, it would be relevant to apply the system for a reverse hybrid approach. In particular, the mRRS system can be used to screen for compounds and drugs that would inhibit the binding between known protein pairs. The ability to perform this screening directly in mammalian cells is a great advantage, since it overcomes drug permeability through the cell membrane and directly tests the toxicity of the compound on

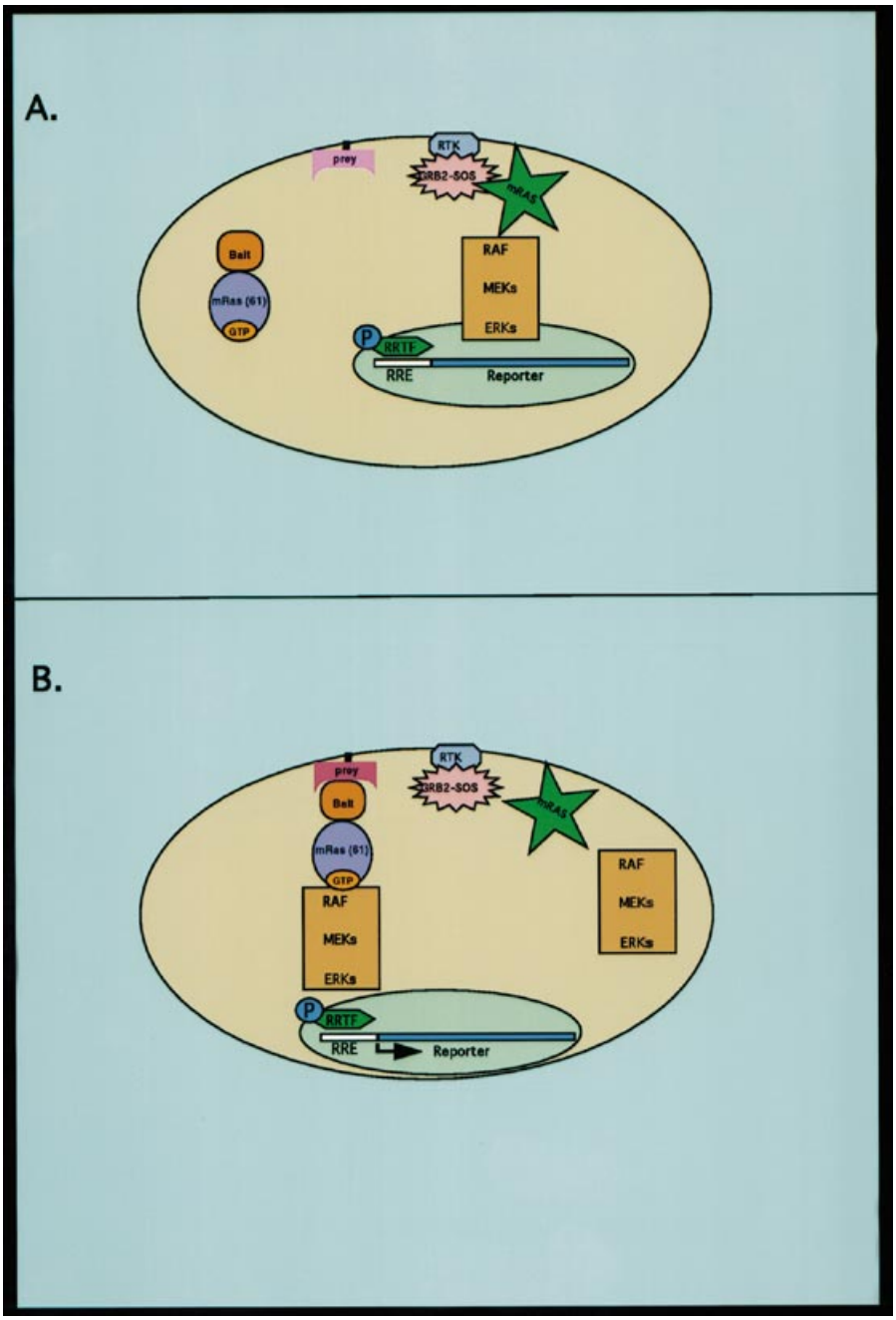

Figure 1. Schematic representation of the mammalian Ras Recruitment assay. (**A**) The endogenous Ras signaling pathway is depicted. Growth factor signals initiate at the membrane receptor tyrosine kinase (RTK) through activation of Grb2-hSos complex to Ras (mRas) and the MAPK cascade resulting in phosphorylation of Ras responsive transcription factors (RRTF) and potentiation of their activity leading to transcription of immediate early genes through DNA elements known as the Ras responsive elements (RRE). Expression of cytoplasmic Ras fused to a 'bait' of interest is not expected to activate the MAPK cascade unless it is located at the plasma membrane. The basal MAPK activity can be monitored using reporter genes under the control of RRE DNA elements. (**B**) Ras membrane localization via protein–protein interaction. In growing cells, the Ras signaling pathway is mildly active. Transfected cytoplasmic Ras fused to a bait protein can be localized to the inner leaflet of the plasma membrane via interaction with a protein partner fused to myristoylation signals. Ras localization results in activation of the MAPK cascade, leading to phosphorylation of RRTF and leading to potentiation of their activity followed by activation of RRE-dependent reporter genes.

the relevant cells. Due to the fact that a variety of compounds may affect the activity of the MAPK cascade, this screening protocol should be carefully designed.

In summary, the mRRS provides a useful and simple tool for the analysis of protein–protein interaction initially identified in yeast, directly in mammalian cells.

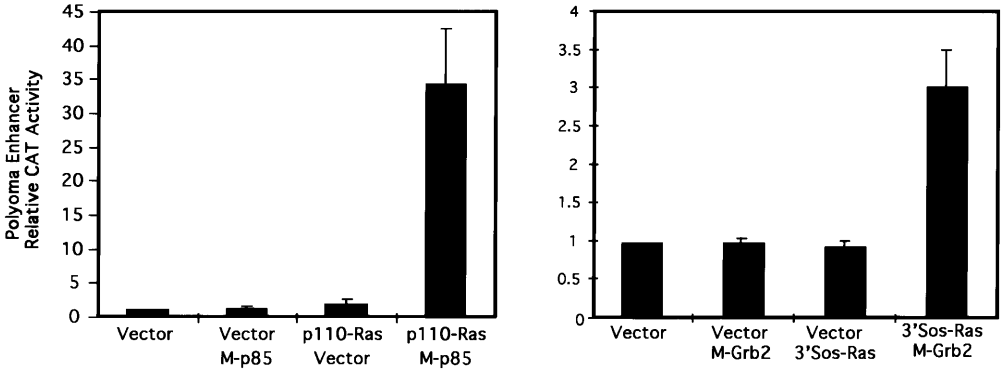

Figure 2. Demonstration of protein–protein interaction between different protein pairs using the polyoma enhancer CAT reporter. Transcription activation of the polyoma enhancer CAT reporter gene via interaction between the catalytic subunit of PI3K (p110) fused to activated cytoplasmic Ras (p110-Ras) and myristoylated PI3K regulatory subunit, p85 (M-p85) (left panel), and between the C-terminal proline-rich domain of hSos fused to Ras (3'Sos-Ras) and myristoylated Grb2 (M-Grb2) (right panel). HEK-293 cells were cotransfected with the indicated plasmids using the calcium phosphate method. The transfection mixture included reporter plasmids (4 μg) and expression plasmids (8 μg). For transfections in which the bait and the prey plasmids were tested alone, total plasmid DNA was kept constant using empty expression plasmid. Forty hours following transfection, cells were harvested and CAT activity was determined. The results are presented as CAT activity relative to the activity obtained by the reporter alone. The results represent the average of four independent experiments.

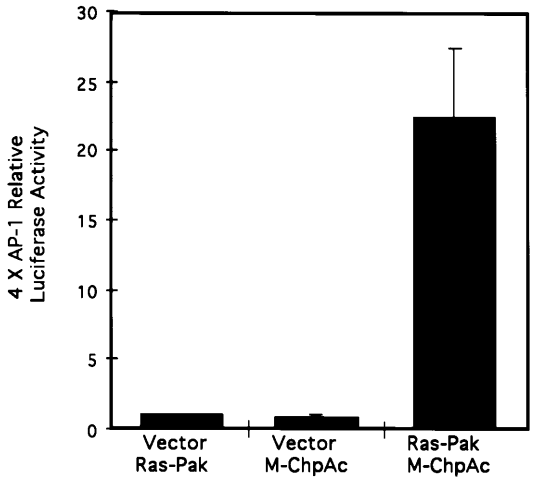

Figure 3. Demonstration of protein–protein interactions between different protein pairs using multiple AP-1 sites linked to a luciferase reporter gene. The interaction between the regulatory subunit of Pak65 fused to cytoplasmic Ras (Ras-Pak) and myristoylated Chp was examined using luciferase reporter plasmid controlled by four copies of AP-1 DNA elements. Cells were transfected as described in Figure 2. The activity obtained with Ras-Pak alone was determined as 100%, and all other activities were calculated respectively. The results represent the average of three independent experiments.

ACKNOWLEDGEMENTS

I wish to thank to Mr Y. C. Broder and Ms S. Katz for fruitful discussions, Ms A. Cohen for excellent technical assistance and Dr Wasylyk Bohdan for the polyoma enhancer CAT-reporter plasmid. This research was supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities–Charles H. Revson Foundation, the Israel Cancer Research Foundation (ICRF). A.A. is a recipient of an academic lectureship from Samuel and Miriam Wein.

REFERENCES

1. Fields,S. and Song,O.K. (1989) *Nature*, **340**, 245–246.
2. Durfee,T., Becherer,K., Chen,P.L., Yeh,S.H., Yang,Y., Kilburn,A.E., Lee,W.H. and Elledge,S.J. (1993) *Genes Dev.*, **7**, 555–569.
3. Estojak,J., Brent,R. and Golemis,E.A. (1995) *Mol. Cell. Biol.*, **15**, 5820–5829.
4. Allen,J.B., Walberg,M.W., Edwards,M.C. and Elledge,S.J. (1995) *Trends Biochem. Sci.*, **20**, 511–516.
5. Boeke,J. and Brachmann,R.K. (1997) *Curr. Biol.*, **8**, 561–568.
6. Brachmann,R.K. and Boeke,J.D. (1997) *Curr. Opin. Biotech.*, **8**, 561–568.
7. Evangelista,C., Lockshon,D. and Fields,S. (1996) *Trends Cell Biol.*, **6**, 196–199.
8. Fredrickson,R.M. (1998) *Curr. Opin. Biotech.*, **9**, 90–96.
9. Hopkin,K. (1996) *J. NIH Res.*, **8**, 27–29.
10. Young,K.H. (1998) *Biol. Reprod.*, **58**, 302–311.
11. Aronheim,A., Zandi,E., Hennemann,H., Elledge,S. and Karin,M. (1997) *Mol. Cell. Biol.*, **17**, 3094–3102.
12. Broder,Y.C., Katz,S. and Aronheim,A. (1998) *Curr. Biol.*, **8**, 1121–1124.
13. Petitjean,A., Higler,F. and Tatchell,K. (1990) *Genetics*, **124**, 797–806.
14. Aronheim,A., Broder,Y.C., Cohen,A., Fritsch,A., Belisle,B. and Abo,A. (1998) *Curr. Biol.*, **8**, 1125–1128.
15. Yu,X., Wu,L.C., Bowcock,A.M., Aronheim,A. and Baer,R. (1998) *J. Biol. Chem.*, **273**, 25388–25392.
16. Wu,L.C., Wang,Z.W., Tsan,J.T., Spillman,M.A., Phung,A., Xu,X.L., Yang,M.-C.W., Hwang,L.-Y., Bowcock,A.M. and Baer,R. (1996) *Nature Genet.*, **14**, 430–440.
17. Dang,C.V., Barrett,J., Villa-Garcia,M., Resar,L.M.S., Kato,G.J. and Fearon,E.R. (1991) *Mol. Cell. Biol.*, **11**, 954–962.
18. Luo,Y.L., Batalao,A., Zhou,H. and Li,Z. (1997) *Biotechniques*, **22**, 350–352.
19. Vasavada,H.A., Ganguly,S., Germino,F.J., Wang,Z.X. and Weissman,S.M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10686–10690.
20. Leanna,C.A. and Hannink,M. (1996) *Nucleic Acids Res.*, **24**, 3341–3347.
21. Vidal,M., Brachmann,R.K., Fattaey,A., Harlow,E. and Boeke,J.D. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 10315–10320.
22. Vidal,M., Braun,P., Chen,E., Boeke,J.D. and Harlow,E. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 10321–10326.
23. Wasylyk,B., Hagman,J. and Gutierrez-Hartmann,A. (1998) *Trends Biochem. Sci.*, **23**, 213–216.
24. Fukunaga,R. and Hunter,T. (1997) *EMBO J.*, **16**, 1921–1933.

25. Krstic,M.D., Rogatsky,I., Yamamoto,K.R. and Garabedian,M.J. (1997) *Mol. Cell. Biol.*, **17**, 3947–3954.
26. Cheng,J.T., Cobb,M.H. and Baer,R. (1993) *Mol. Cell. Biol.*, **13**, 801–808.
27. Wasylyk,C., Imler,J.L., Perez-Mutul,J. and Wasylyk,B. (1987) *Cell*, **48**, 525–534.
28. Aronheim,A., Engelberg,D., Li,N., Al-Alawi,N., Schlessinger,J. and Karin,M. (1994) *Cell*, **78**, 949–961.
29. Yoshioka,K., Deng,T., Cavigelli,M. and Karin,M. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 4972–4976.
30. Hancock,J.F., Magee,A.I., Childs,J. and Marshall,C.J. (1989) *Cell*, **57**, 1167–1177.
31. Leevers,S.J., Paterson,H.F. and Marshall,C.J. (1994) *Nature*, **369**, 411–414.
32. Carpenter,C.L. and Cantley,L.C. (1996) *Curr. Opin. Cell Biol.*, **8**, 153–158.
33. Manser,E., Leung,T., Salihuddin,H., Zhao,Z.S. and Lim,L. (1994) *Nature*, **367**, 40–46.
34. Brown,P.H., Chen,T.K. and Birrer,M.J. (1994) *Oncogene*, **9**, 791–799.

Figure 1. Schematic representation of the mammalian Ras Recruitment assay. (**A**) The endogenous Ras signaling pathway is depicted. Growth factor signals initiate at the membrane receptor tyrosine kinase (RTK) through activation of Grb2-hSos complex to Ras (mRas) and the MAPK cascade resulting in phosphorylation of Ras responsive transcription factors (RRTF) and potentiation of their activity leading to transcription of immediate early genes through DNA elements known as the Ras responsive elements (RRE). Expression of cytoplasmic Ras fused to a 'bait' of interest is not expected to activate the MAPK cascade unless it is located at the plasma membrane. The basal MAPK activity can be monitored using reporter genes under the control of RRE DNA elements. (**B**) Ras membrane localization via protein–protein interaction. In growing cells, the Ras signaling pathway is mildly active. Transfected cytoplasmic Ras fused to a bait protein can be localized to the inner leaflet of the plasma membrane via interaction with a protein partner fused to myristoylation signals. Ras localization results in activation of the MAPK cascade, leading to phosphorylation of RRTF and leading to potentiation of their activity followed by activation of RRE-dependent reporter genes.

Figure 2. Demonstration of protein–protein interaction between different protein pairs using the polyoma enhancer CAT reporter. Transcription activation of the polyoma enhancer CAT reporter gene via interaction between the catalytic subunit of PI3K (p110) fused to activated cytoplasmic Ras (p110-Ras) and myristoylated PI3K regulatory subunit, p85 (M-p85) (left panel), and between the C-terminal proline-rich domain of hSos fused to Ras (3'Sos-Ras) and myristoylated Grb2 (M-Grb2) (right panel). HEK-293 cells were cotransfected with the indicated plasmids using the calcium phosphate method. The transfection mixture included reporter plasmids (4 μg) and expression plasmids (8 μg). For transfections in which the bait and the prey plasmids were tested alone, total plasmid DNA was kept constant using empty expression plasmid. Forty hours following transfection, cells were harvested and CAT activity was determined. The results are presented as CAT activity relative to the activity obtained by the reporter alone. The results represent the average of four independent experiments.

Figure 3. Demonstration of protein–protein interactions between different protein pairs using multiple AP-1 sites linked to a luciferase reporter gene. The interaction between the regulatory subunit of Pak65 fused to cytoplasmic Ras (Ras-Pak) and myristoylated Chp was examined using luciferase reporter plasmid controlled by four copies of AP-1 DNA elements. Cells were transfected as described in Figure 2. The activity obtained with Ras-Pak alone was determined as 100%, and all other activities were calculated respectively. The results represent the average of three independent experiments.