Cell culture, reporter assay and expression of human BRM and Brg1 in insect cell

293T, SW13 and C33A cells were maintained in Dulbecco's modified Eagle's medium (DMEM). Cells were grown supplemented with 10% fetal calf serum. G401 cell was cultured in RPMI medium-1640 (Invitrogen) supplemented with 10% calf serum. Transfections of all expression vectors, pG5luc and pRL-TK, were carried out by using Lipofectamine Plus reagent (Invitrogen). After 36 h of transfection, the cells in 24-well plates were washed by phosphate-buffered saline (PBS) and analysed for luciferase activities in triplicate in each transfection experiment by using Dual-Luciferase Reporter Assay System (Promega). Sf9 insect cell was grown in Grace's Insect Medium (Invitrogen) supplemented with 10% fetal calf serum. Expression procedure of human BRM or Brg1 in Sf9 insect cell was performed using Bac-to-Bac Baculovirus Expression System (Invitrogen).

GST pull-down assay

GST-Gal4-SYT mutant DNAs were transformed in BL21 pLys-S (Novagen). Bacterial cells that expressed GST fusion proteins were harvested and re-suspended in buffer A [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 500 mM NaCl, protease inhibitors]. After sonication, the lysates were centrifuged for 30 min at 8000 rpm. The supernatants were incubated with glutathione sepharose 4B beads (Amersham Pharmacia Biotech) at 4 °C for 1 h and the beads were washed with buffer A for four times. The Sf9 cells expressed flag tagged BRM or Brg1 were washed with PBS and resuspended in buffer B [10 mM Tris-HCl (pH 8.0), 10 mM glycerol, 0.15 M NaCl, 0.1% nonidet P-40, 5 mM 2-mercaptoethanol, protease inhibitors]. After sonication, the lysates were centrifuged for 30 min at 15 000 rpm. The supernatants were incubated with 30 μL of anti- flag M2 Agarose (Sigma) at 4 °C for overnight and washed five times with buffer B. Bound proteins were eluted with buffer B containing 0.2 mg/mL of flag peptide (Sigma). The elutes were incubated with GST-beads immobilized GST-Gal4-SYT mutants in buffer B at 4 °C for 4 h. The beads were washed four times with buffer B without 2-mercaptoethanol and re-suspended in sample buffer. The bound proteins were separated by 8% SDS–PAGE (sodium dodecyl sulfate—polyacrylamide gel electrophoresis) and analysed with anti-BRM (sc-6450, Santa Cruz) and anti-Brg1 (sc-8749, Santa Cruz) antibodies, respectively. The ECL System (Amersham Pharmacia Biotech) was used for detection on RX-U X-ray films (Fuji).

Immunoprecipitation

The expression vectors of flag-tagged SYT mutants (without GAL4 DNA binding domain), no-tagged BRM and Brg1 were transfected to 293T by Lipofectamine Plus reagent. After 36 h, the cells were washed and suspended in NP-40 lysis buffer [10 mM Tris-HCl (pH 7.8), 1% nonidet P-40, 0.15 M NaCl, 1 mM EDTA, protease inhibitors]. Then lysates were centrifuged for 30 min at 15 000 rpm. The supernatants were incubated with 30 μL of anti-flag M2 agarose at 4 °C for overnight and washed four times with NP-40 lysis buffer. The bound proteins were detected by Western blot.

Immunofluorescence microscopy

Expression vectors of GFP-SYT-WT, HA-tagged BRM or Brg1 were transfected in SW13 using the same method of immunoprecipitaion. After 24 h, the cells were removed to a chamber slide, incubated a further 24 h, fixed with 3% formaldehyde in PBS for 10 min. After washing by PBS, the cells were permeabilized with 0.1% polyoxyethylene octylphenyl ether (10) in PBS for 5 min and washed with PBS. The slide was incubated in blocking buffer (2% normal swine serum in PBS) for 20 min. Cells were incubated overnight with primary antibody at 4 °C, washed three times with PBS and incubated with secondary antibody for 40 min at 25 °C. As primary antibodies, we used anti-BRM (sc-6450 and sc-6449, Santa Cruz), anti-Brg1 (sc-8749, Santa Cruz). As secondary antibodies, we used rhodamine-conjugated donkey anti-goat (sc-2094, Santa Cruz). Finally, the slide was mounted with DAPI and visualized under Leica DM IBD microscope with appropriate filters and images were acquired by Leica Qfluoro.

Acknowledgements

We thank Drs Takashi Obinata (Chiba University), Hideo Iba (University of Tokyo) and Susumu Hirose (National Institute of Genetics of Japan) for valuable suggestions and for providing the SW13 cell. This work was supported in part by the programme for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Brett, D., Whitehouse, S., Antonson, P., Shipley, J., Cooper, C. & Goodwin, G. (1997) The SYT protein involved in the t(X;18) synovail sarcoma translocation is a transcriptional activator localised in nuclear bodies. *Hum. Mol. Genet.* **6**, 1559–1564.

de Bruijn, D.R.H., dos Santos, N.R., Thijssen, J., *et al.* (2001a) The synovial sarcoma associated protein SYT interacts with the acute leukemia associated protein AF10. *Oncogene* **20**, 3281–3289.

de Bruijn, D.R.H., Kater-Baats, E., Eleveld, M., Merkx, G. & Geurts van Kessel, A. (2001b) Mapping and characterization of the mouse and human SS18 genes, two human SS18-like genes and a mouse Ss18 pseudogene. *Cytogenet. Cell. Genet.* **92**, 310–319.

Clark, J., Rocques, P.J., Crew, A.J., *et al.* (1994) Identification of novel genes, *SYT* and *SSX*, involved in the t(X;18) (p11.2;q11.2) translocation found in human synovial sarcoma. *Nat. Genet.* **7**, 502–508.

Crew, A.J., Clark, J., Fisher, C., *et al.* (1995) Fusion of *SYT* to two genes, *SSX1* and *SSX2*, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma. *EMBO J.* **14**, 2333–2340.

DeCristofaro, M.F., Betz, B.L., Wang, W. & Weissman, B.E. (1999) Alteration of hSNF5/INI1/BAF47 detected in rhabdoid cell lines and primary rhabdomyosarcomas but not Wilms' tumors. *Oncogene* **18**, 7559–7565.

DeCristofaro, M.F., Betz, B.L., Rorie, C.J., *et al.* (2001) Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. *J. Cell. Physiol.* **186**, 136–145.

Eid, J.E., Kung, A.L., Scully, R. & Livingston, D.M. (2000) p300 interacts with the nuclear proto-oncoprotein SYT as part of the active control of cell adhesion. *Cell* **102**, 839–848.

Gure, A.O., Tureci, O., Sahin, U., *et al.* (1997) *SSX*: A multigene family with several members transcribed in normal testis and human cancer. *Int. J. Cancer* **72**, 965–971.

Khavari, P.A., Peterson, C.L., Tamkun, J.W., Mendel, D.B. & Crabtree, G.R. (1993) BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature* **366**, 170–174.

Kornberg, R.D. & Lorch, Y. (1999) Twenty-five years of the nucleosomes, fundamental particle of the eukaryote chromosome. *Cell* **98**, 285–294.

Kuzmichev, A., Zhang, Y., Erdjument-Bromage, H., Tempst, P. & Reinberg, D. (2002) Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor $p33^{ING1}$. *Mol. Cell. Biol.* **22**, 835–848.

Lim, F.L., Soulez, M., Koczan, D., Thiesen, H.J. & Knight, J.C. (1998) A KRAB-related domain and a novel transcription repression domain in proteins encoded by SSX genes that are disrupted in human sarcomas. *Oncogene* **17**, 2013–2018.

Muchardt, C., Reyes, J.-C., Bourachot, B., Legouy, E. & Yaniv, M. (1996) The hbrm and BRG-1 proteins, components of human SNF/SWI complex, are phosphorylated and excluded from the condensed chromosomes during mitosis. *EMBO J.* **15**, 3394–3402.

Muchardt, C. & Yaniv, M. (1993) A human homologue of *Saccharomyces cerevisiae SNF2/SWI2* and *Drosophila brm* genes potentiates transcriptional activation by the glucocorticoid receptor. *EMBO J.* **12**, 4279–4290.

Muchardt, C., Sardet, C., Bourachot, B., Onufryk, C. & Yaniv, M. (1995) A human protein with homology to *Saccharomyces cerevisiae SNF5* interacts with the potential helicase hbrm. *Nucl. Acids Res.* **23**, 1127–1132.

Murphy, D.J., Hardy, S. & Engel, D.A. (1999) Human SWI-SNF component BRG1 represses transcription of the *c-fos* gene. *Mol. Cell. Biol.* **19**, 2724–2733.

Nagai, M., Tanaka, S., Tsuda, M., *et al.* (2001) Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2α. *Proc. Natl Acad. Sci. USA* **98**, 3843–3848.

Pawson, T. (1995) Protein modules and signaling networks. *Nature* **373**, 573–580.

dos Santos, N.R., de Bruijn, D.R.H. & van Kessel, A.G. (2001) Molecular mechanisms underlying human synovial sarcoma development. *Genes, Chromosomes Canc.* **30**, 1–14.

Stern, M., Jensen, R. & Herskowitz, I. (1984) Five *SWI* genes are required for expression of the *HO* gene in yeast. *J. Mol. Biol.* **178**, 853–868.

Storlazzi, C.T., Mertens, F., Mandahl, N., *et al.* (2003) A novel fusion gene, SS18L1/SSX1, in synovial sarcoma. *Genes, Chromosomes Canc.* **37**, 195–200.

Strobeck, M.W., Knudsen, K.E., Fribourg, A.F., *et al.* (2000) BRG-1 is required for RB-mediated cell cycle arrest. *Proc. Natl. Acad. Sci. USA* **97**, 7748–7753.

Thaete, C., Brett, D., Monaghan, P., *et al.* (1999) Functional domains of SYT and SYT-SSX synovial sarcoma translocation proteins and co-localization with the SNF protein BRM in the nucleus. *Hum. Mol. Genet.* **8**, 585–591.

Turc-Carel, C., Dal Cin, P., Limon, J., *et al.* (1987) Involvement of chomosome X in primary cytogenetic change in human neoplasia: nonrandom translocation in synovial sarcoma. *Proc. Natl. Acad. Sci. USA* **84**, 1981–1985.

Versteege, I., Sevenet, N., Lange, J., *et al.* (1998) Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* **394**, 203–206.

Vignali, M., Hassan, A.H., Neely, K.E. & Workman, J.L. (2000) ATP-dependent chromatin—remodeling complexes. *Mol. Cell. Biol.* **20**, 1899–1910.

Wang, W., Cote, J., Xue, Y., *et al.* (1996) Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *EMBO J.* **15**, 5370–5382.

Zhang, H.S., Gavin, M., Dahiya, A., *et al.* (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101**, 79–89.

Received: 6 November 2003
Accepted: 12 February 2004