An essential role of ERK signalling in TPA-induced reactivation of Kaposi’s sarcoma-associated herpesvirus

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Kaposi’s sarcoma-associated herpesvirus (KSHV) is implicated causally in the development of several human malignancies, including primary effusion lymphoma (PEL). PEL cells serve as tools for KSHV research, as most of them are latently infected and allow lytic virus replication in response to various stimuli. 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) is the most potent inducer of lytic KSHV reactivation; nevertheless, the exact mechanism by which it induces reactivation remains unknown. It has previously been reported by our group that the protein kinase C (PKC) δ isoform plays a crucial role in TPA-mediated KSHV reactivation. Here, the activation pathway was dissected and it was demonstrated that TPA induces KSHV reactivation via stimulation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. Western blot analysis revealed a rapid phosphorylation of ERK1/2. Cells treated with MAPK/ERK inhibitors before TPA addition demonstrated repression of ERK1/2 phosphorylation, which was associated with a block of KSHV lytic-gene expression. This inhibition prevented c-Fos accumulation, yet increased c-Jun phosphorylation. Similar results were obtained in response to rottlerin, a selective PKCδ inhibitor. Notably, the PKC inhibitor GF 109203X reduced ERK1/2 phosphorylation, c-Fos accumulation, c-Jun phosphorylation and KSHV reactivation. It is proposed that TPA induces KSHV reactivation through at least two arms. The first involves PKCδ, ERK phosphorylation and c-Fos accumulation, whilst the second requires another PKC isoform that induces the phosphorylation of c-Jun. c-Fos and c-Jun jointly form an active AP-1 complex, which functions to activate the lytic cascade of KSHV.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) is a human gammaherpesvirus that is linked aetiologically to the development of Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL; also known as body cavity-based lymphoma) and a subset of multicentric Castleman’s disease (Chang et al., 1994; Dourmishev et al., 2003; Cohen et al., 2005). Primary infection with KSHV persists for life, but in most cases never leads to disease development. In the host cell, infection with KSHV displays two distinct cycles of DNA replication and gene expression: latent and lytic. During latency, the viral genome persists as a circular DNA episome, only few viral genes are expressed and no viral particles are produced. In contrast, extensive viral DNA replication and a well-controlled array of viral-gene expression characterize the lytic phase, which may end in the assembly and release of new viral particles. The lytic virus-replication cycle is crucial for virus spread between cells and hosts, and is also likely to play an important role in the tumorigenesis induced by KSHV (Grundhoff & Ganem, 2004; Krishnan et al., 2004; Naranatt et al., 2004). Certain physiological conditions, which are poorly defined to date, reactivate the hidden latent virus periodically in most asymptomatic carriers, potentially leading to disease onset. The key viral protein necessary for the initiation of the lytic cycle is the replication and transcription activator (Rta), encoded by open reading frame (ORF) 50 (West & Wood, 2003). The stimulation of KSHV lytic-gene expression by Rta employs several mechanisms and involves interactions with selected cellular transcription factors (Sakakibara et al., 2001; Wang et al., 2001, 2004; Gwack et al., 2002; Liang et al., 2002; Haque et al., 2003; Lan et al., 2005; Liang & Ganem, 2003; West & Wood, 2003). Nevertheless, it is clear that certain, yet inadequately understood, host signal-transduction pathways operate to initiate the switch between latency and productive infection.

Cell lines that were established from PEL patients harbour multiple episodes of KSHV and serve as valuable tools for KSHV research (Boshoff et al., 1998; Cesarman et al., 1995; Renne et al., 1996). Under standard growth conditions, PEL cells remain predominantly latently infected by KSHV, whilst only a small, but steady, fraction express lytic viral proteins and produce new viruses. An increased, but limited, lytic virus replication can be induced in these cells by various stimuli, including Rta overexpression (Bechtel et al., 2003;
West & Wood, 2003), co-infection with another virus (Varthakavi et al., 1999; Vieira et al., 2001), hypoxic conditions (Davis et al., 2001), interleukin 6 (IL-6) (Chang et al., 2000; Chatterjee et al., 2002; Song et al., 2002), gamma interferon (Blackbourn et al., 2000; Chang et al., 2000; Mercader et al., 2000) and chemical agents, such as n-butyrat (Miller et al., 1996), ionomycin (Chang et al., 2000; Zoeteweij et al., 2001) and 5-azacytidine (Chen et al., 2001). Nonetheless, the most potent stimulus for KSHV reactivation in the majority of PEL cells, as well as in other experimentally KSHV-infected cell lines, is the protein kinase C (PKC) agonist 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Moore et al., 1996; Renne et al., 1996). The mechanisms and signal-transduction pathways that are initiated by TPA, leading to Rta activation and, consequently, to KSHV reactivation, are at the focus of the present study.

Signals transmitted through PKC can potentially activate the network of mitogen-activated protein kinase (MAPK) signal-transduction pathways, which play an important role in regulating the response of cells to various extracellular stimuli, such as TPA (Rubinfeld & Seger, 2004; Kolch et al., 2005). The MAPK signalling pathways are activated by sequential phosphorylation steps, through five pathways designated the extracellular signal-regulated kinase (MAPK/ERK or ERK1/2), the stress-activated protein kinase/c-Jun N-terminal protein kinase (SAPK/JNK), p38 MAPK, ERK5 and ERK3/4. Signals triggering the MAPK/ERK pathway are normally transmitted to the MAPK kinase kinase, c-Raf1, directing the phosphorylation of one or both isoforms of the MAPK kinases, MEK1 and MEK2 (MEK1,2), which then phosphorylate one or both of the MAPK/ERK components, p44 ERK1 and p42 ERK2 (ERK1/2). In response to phosphorylation, ERK1/2 translocate from the cytoplasm to the nucleus and activate a variety of targets, including specific protein kinases and transcription factors, such as c-Fos. Recently, the cellular activator protein-1 (AP-1) complex, formed by dimerization of c-Jun and c-Fos, was reported to activate early-lytic KSHV promoters, among them the Rta promoter, in PEL cells (Wang et al., 2004).

We have previously reported the necessity of the δ isoform of PKC during TPA-stimulated lytic reactivation of KSHV (Deutsch et al., 2004). Here, we studied this signalling cascade further and describe the critical role of the MAPK/ERK pathway in the events resulting in KSHV lytic reactivation. We present data indicating the initiation and involvement of at least two cellular signal-transduction pathways during KSHV reactivation by TPA; the first involves PKCδ, ERK and c-Fos activation, whilst the second requires a distinct PKC isoform that leads to c-Jun phosphorylation.

RESULTS

TPA induces ERK1/2 phosphorylation

To determine whether the MAPK/ERK signal-transduction pathway is a likely participant in the events leading to KSHV reactivation by TPA, we first tested the phosphorylation of ERK1/2 at different time points following TPA stimulation. As shown in Fig. 1, ERK1/2 phosphorylation was induced by TPA as early as 5 min after stimulation and continued to increase up to 24 h following stimulation. In addition, results shown in Fig. 1 demonstrate the preferential phosphorylation of ERK2 following TPA stimulation (lower band in doublet); this observation was consistent throughout our entire study. Immunoblotting with antibody against ERK1, which also detects ERK2, revealed that the total level of these proteins remained unchanged. Of note, the highest

METHODS

Cell-culture conditions. The KSHV-infected, PEL-derived cell line, BCP-1 (Booshoff et al., 1998), was maintained at 37 °C in RPMI 1640 medium (Gibco) containing 2 mM l-glutamine and 10 % fetal calf serum (Biological Industries) in the presence of 5 % CO₂. To induce lytic virus reactivation, cells were subcultured at 2 x 10⁵ cells ml⁻¹, incubated overnight and exposed to 20 ng TPA ml⁻¹ (32 nM) (Sigma). To test the effect of PKC inhibitors, cells were subcultured as described above and treated with either 5 μM GF109203X (bis-indolylmaleimide I) or 5 μM rottlerin (Calbiochem) 30 min prior to the addition of TPA. For inhibition of ERK1/2 phosphorylation, cells were treated with 50 μM of the MEK1,2 inhibitors PD98059 or U0126 (Sigma) at the indicated time points prior to or following TPA addition.

Immunoblot analysis. Cells were washed twice in cold PBS, suspended in lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % Nonidet P-40, 0.1 % SDS, 1 mM PMSF, 50 μg aprotinin ml⁻¹, 50 μM leupeptin, 0.2 mM NaVO₄, 50 mM NaF] and incubated on ice for 30 min. Cell debris was then removed by centrifugation at 12 000 g for 15 min at 4 °C. Loading buffer [2 x ; 2 % SDS, 20 % glycerol, 125 mM Tris (pH 6.8), 0.02 % bromophenol blue and 10 % β-mercaptoethanol] was added and the samples were boiled for 5 min. Protein lysates were resolved by SDS-PAGE and were transferred to nitrocellulose membranes (Schleicher & Schuell). Similar protein contents of the different samples were verified by Ponceau staining. The nitrocellulose membranes were blocked with 5 % dried milk in TBS or 1 % dried milk and 1 % BSA in TBS and subsequently incubated with primary antibody. Specific reactive bands were detected by using goat anti-rabbit IgG or goat anti-mouse conjugated to horseradish peroxidase (Bio-Rad; Jackson ImmunoResearch Laboratories, Inc.). Immunoactive bands were visualized by an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences). Antibodies against phospho-ERK, ERK1, ERK2, phospho-c-Jun and c-Fos were obtained from Santa Cruz Biotechnology Inc. Anti-KSHV viral IL-6 (vIL-6) was kindly provided by Professors P. Moore and Y. Chang (University of Pittsburgh, PA, USA) and monoclonal anti-Rta antibody was kindly provided by Dr Keiji Ueda (Osaka University School of Medicine, Osaka, Japan).

Adenovirus preparation and infection. The AdEasy system was kindly provided by Professor B. Vogelstein (The Johns Hopkins University School of Medicine, MD, USA) (He et al., 1998). PKCδ and PKCα K376R kinase-defective mutant (Blass et al., 2002) were first cloned into pShuttle–CMV vector. These plasmids were then linearized by digestion with Pmel and transformed into Escherichia coli BJ15183-AD-1 competent cells (Strategene) carrying the pAdEasy-1 plasmid that encodes the adenovirus 5 backbone. Recombination was confirmed by restriction and PCR analyses. The linearized recombinant plasmids were transfected into HEK293 cells and, after 10 days, viruses were collected and further amplified. End-point dilution assay was used to determine titres of the adenoviral stocks.
level of ERK1/2 phosphorylation was evident after 24 h TPA stimulation. Taken together, these findings indicate that ERK is activated by TPA and hence may participate in the signalling pathways that lead to virus reactivation in response to this stimulus. Furthermore, as ERK phosphorylation persisted, the MAPK/ERK pathway may play a role not only in triggering lytic reactivation, but also during subsequent virus-production activities.

Inhibition of the MAPK/ERK pathway blocks KSHV reactivation

To determine whether the MAPK/ERK pathway is important for KSHV lytic reactivation, we investigated the effects of two chemical inhibitors of this pathway, PD98059 and U0126. PD98059 is known to inhibit MEK1 and, to a lesser extent, MEK2 (Alessi et al., 1995; Dudley et al., 1995). U0126 prevents the activation of MEK1,2 by upstream Raf kinase and also blocks the catalytic activity of pre-existing activated MEK1,2 and, hence, its ability to activate downstream MAPK/ERK (Favata et al., 1998). BCP-1 cells were treated with PD98059 or U0126 for the indicated time points prior to or after TPA addition and collected after 24 h. As previously reported, KSHV lytic reactivation was induced following TPA stimulation (Moore et al., 1996; Renne et al., 1996; Sarid et al., 1998; Deutsch et al., 2004). This was evident by the induction of the expression of the early-lytic protein vIL-6 24 h after stimulation (Fig. 2). Partial inhibition of ERK phosphorylation was evident when 50 μM PD98059 was added prior to the addition of TPA. However, this was sufficient to decrease virus reactivation, as measured by vIL-6 antibodies (Fig. 2a). U0126 exhibited a higher level of inhibition of TPA-induced ERK phosphorylation, perhaps due to an equally inhibitory effect on MEK1 and MEK2, resulting in the complete blockage of KSHV reactivation (Fig. 2b). The inhibition of virus reactivation in cells that were stimulated by TPA after treatment with the inhibitors probably accounts for the prevention of MAPK/ERK signalling at an early phase of the TPA cascade. Of note, addition of MEK1,2 inhibitors 4 h after stimulation by TPA also resulted in decreased vIL-6 expression, indicating a possible role of the MAPK/ERK signalling pathway during further productive viral functions in cells that have already entered the lytic cycle (Fig. 2a, b). These results are consistent with the hypothesis that KSHV lytic reactivation by TPA depends to a large extent on the activity of the MAPK/ERK pathway.

c-Fos, but not phospho-c-Jun, is regulated by MAPK/ERK signalling

The responsiveness of the viral Rta promoter to the AP-1 complex, formed by c-Jun and c-Fos heterodimers, has already been established (Wang et al., 2004). In addition, induction of c-Jun phosphorylation as early as 1 h following TPA stimulation of PEL cells has been reported (Wang et al., 2004). To investigate the relationship between MAPK/ERK activation and the accumulation of c-Fos and phospho-c-Jun, BCP-1 cells were first treated with the MAPK/ERK inhibitor U0126 for 30 min and then TPA was added for 2, 4 or 24 h. Untreated cells and cells treated with TPA for similar time periods served as controls. As expected, treatment with TPA induced Rta expression and a rapid accumulation of c-Fos at 2 h, peaking at 4 h and declining after 24 h. MAPK/ERK inhibition blocked this response completely (Fig. 3). In contrast, MAPK/ERK inhibition did not reduce the phosphorylation of c-Jun, which was also evident at all time points after exposure to TPA, and c-Jun
instead demonstrated a time-dependent increase of its signal. These findings suggest that MAPK/ERK signalling is required for the activation of c-Fos, whilst its partner in the AP-1 complex, c-Jun, is stimulated by another signal-transduction pathway.

**PKC inhibition diminishes ERK phosphorylation and KSHV reactivation**

One of the signalling pathways induced by PKC involves the activation of the MAPK/ERK pathway (Kolch et al., 2005). We have shown previously that TPA-mediated KSHV reactivation is largely decreased by inhibition of the PKC\(\delta\) isoform (Deutsch et al., 2004). To evaluate the relationship between ERK phosphorylation and PKC activation during TPA-stimulated KSHV reactivation, we examined the phosphorylation of ERK1/2 in cells that were treated with PKC inhibitors prior to the addition of TPA. In line with previous findings (Deutsch et al., 2004), treatment with 5 \(\mu\)M GF 109203X, which inhibits the \(\alpha\), \(\beta_{1}\), \(\beta_{2}\), \(\gamma\), \(\delta\) and \(\varepsilon\) PKC isoforms (Toullec et al., 1991), as well as with 5 \(\mu\)M rottlerin, a selective inhibitor of PKC\(\delta\) (Gschwendt et al., 1994), resulted in a large reduction of ERK1/2 phosphorylation in response to TPA, coupled with a compatible decrease in the expression of vIL-6 (Fig. 4). Similar results were obtained by the use of a recombinant adenoviral vector expressing a mouse kinase-defective K376R PKC\(\delta\) mutant (Blass et al., 2002). BCP-1 cells were incubated 24 h after adenoviral transduction, exposed to TPA for an additional 24 h and then either treated with TPA or left untouched. Transduction of empty adenovirus vector (CV + TPA). These results suggest that the MAPK/ERK pathway is a downstream effector of PKC\(\delta\).

**Inhibition of PKC by GF 109203X prevents c-Fos and c-Jun activation, whereas inhibition of PKC\(\delta\) decreases c-Fos accumulation, but not c-Jun phosphorylation**

As PKC inhibition by GF109203X blocked ERK1/2 phosphorylation and vIL-6 induction, whilst MAPK/ERK inhibition reduced c-Fos but not c-Jun, we examined the role of PKC in c-Fos and c-Jun activation at early (2, 4 h) and late (24 h) TPA stimulation alone or with the empty adenovirus control vector (CV + TPA). These results suggest that the MAPK/ERK pathway is a downstream effector of PKC\(\delta\).
later (24 h) time points post-TPA induction. As shown in Fig. 6(a), treatment with GF 109203X prior to TPA stimulation resulted in a concomitant inhibition of Rta induction, ERK and c-Jun phosphorylation and c-Fos accumulation at all time points. Further examination of the role of PKCδ revealed that treatment with rottlerin prior to stimulation by TPA resulted in a large reduction of ERK1/2 phosphorylation and c-Fos accumulation, along with a time-dependent increase in c-Jun phosphorylation (Fig. 6b). Taken together, these results are consistent with the above-described findings and indicate that TPA-mediated KSHV reactivation involves PKCδ, ERK1/2 phosphorylation and c-Fos accumulation. In parallel, TPA induces the phosphorylation of c-Jun through a distinct pathway that requires neither PKCδ activation nor ERK1/2 phosphorylation, but involves an as-yet-unidentified PKC isoform. Furthermore, the inhibition of selected components involved in the c-Fos activation pathway results in a considerable shift of TPA signalling towards the c-Jun activation pathway; however, this pathway is not sufficient to induce KSHV reactivation by itself. Thus, it is likely that TPA induces at least two signalling pathways concomitantly, both involving PKC, although only one leads to MAPK/ERK activation.

DISCUSSION

Previously, we reported that the δ isoform of PKC plays an essential role in PEL cells during stimulation of KSHV reactivation by TPA (Deutsch et al., 2004). To further characterize this signalling mechanism, we focused specifically on the relationship between PKC, the MAPK/ERK pathway and KSHV reactivation. Here, we establish that ERK1/2 are phosphorylated upon stimulation by TPA and demonstrate that inhibition of the MAPK/ERK pathway results in the prevention of ERK1/2 phosphorylation and the accumulation of c-Fos, along with a blockage of the lytic reactivation of KSHV. In line with these findings, inhibition of the activity of PKC isoforms with GF 109203X decreased the phosphorylation of ERK1/2 and the accumulation of c-Fos and also abolished KSHV reactivation. Similar results were obtained when the activity of the δ isoform of PKC was repressed experimentally. On the other hand, activation of c-Jun, shown to be a necessary participant in an active AP-1 complex in PEL cells (Wang et al., 2004), did not depend on PKCδ or on ERK1/2 activity. c-Jun phosphorylation required the activity of a yet-to-be-defined classical or novel PKC isoform. Of note, an increased level of c-Jun phosphorylation was evident in cells treated with rottlerin prior to TPA stimulation, suggesting that the blockage of PKCδ activation shifts the signalling pathway to induce the phosphorylation of c-Jun preferentially.

Taken together, our data suggest the following: (i) activation of the MAPK/ERK signalling pathway is critical for disruption of KSHV latency by TPA; (ii) the induction of KSHV reactivation occurs downstream of the phosphorylation of ERK1/2; (iii) the phosphorylated ERK1/2 may participate in further activation pathways mediated by the viral Rta protein; (iv) accumulation of c-Fos depends on ERK1/2 activation; (v) induction of c-Jun phosphorylation by TPA does not depend on the MAPK/ERK pathway; (vi) PKCδ is an important mediator of ERK1/2 phosphorylation and c-Fos accumulation; (vii) an alternative PKC isoform is probably involved in the induction of c-Jun phosphorylation. These data are in line with our previous finding, which demonstrated that overexpression of PKCδ was not sufficient to induce lytic reactivation of KSHV.
Fig. 7. Proposed model for the signalling pathway activated by TPA in PEL cells and required for KSHV reactivation. TPA concomitantly induces at least two signalling pathways required for lytic reactivation of KSHV. The first involves the PKCβ isoform, leading to MAPK/ERK activation and c-Fos accumulation. The second pathway requires another PKC isoform, which induces the phosphorylation of c-Jun. c-Fos and c-Jun together form an active AP-1 complex required for activation of the Rta promoter. In addition, the MAPK/ERK pathway is probably involved in subsequent functions of the lytic activation cascade.

(Deutsch et al., 2004). Fig. 7 illustrates our proposed model for TPA-induced KSHV reactivation.

The classic view of KSHV reactivation describes a coordinately controlled cascade of viral-gene expression during lytic reactivation (Sarid et al., 1998; Sun et al., 1999; Zhu et al., 1999; Jenner et al., 2001). In view of the virus–cell interactions, virus reactivation can further be dichotomized into two stages. The first involves cellular events that trigger virus reactivation, occur prior to the primary activation of the Rta promoter and spark Rta expression. The second includes direct and indirect activation of the expression of lytic viral genes by Rta, leading to viral particles production and release. Currently, distinction between events of the two stages is difficult, as the initial triggering of virus reactivation induces low expression of Rta, which in turn is positively autoregulated and further transcribed. As cycloheximide treatment, which could allow differentiation between path- way events involved during the two stages, results in cell death in BCP-1 cells, avoidance of Rta translation by using this compound is not feasible. In addition, transcription of Rta at the initial stage could be below levels that can be detected and quantified. Nonetheless, the model presented in Fig. 7, demonstrating a responsiveness of the Rta promoter to an active AP-1 complex composed of c-Jun and c-Fos, is consistent with previously published reports (Wang et al., 2004). It should be mentioned that involvement of the MAPK/ERK pathway following primary activation of KSHV is in accordance with previous reports describing a role for the MAPK/ERK pathway in the activation of early cytomegalovirus (CMV) promoters (Rodems & Spector, 1998; Chen & Stinski, 2002). A similar model whereby phorbol ester activates PKC signals through MEK1,2 to induce expression of the immediate-early transcription activator BZLF1 has been proposed for EBV (Fenton & Sinclair, 1999).

Other viruses have evolved to take advantage of selected cellular MAPK signal-transduction pathways to regulate their entry into cells and to alter viral- and cellular-gene expression. HSV-1 infection activates p38 MAPK and SAPK/JNK (McLean & Bachenheimer, 1999; Zachos et al., 1999). Adenovirus and Hepatitis B virus activate the MAPK/ERK and p38 MAPK pathways following infection (Benn et al., 1996; Bruder & Kovesdi, 1997; Suomalainen et al., 2001) and CMV activates the MAPK/ERK or p38 MAPK pathways at early times after infection (Chen et al., 2002). Human immunodeficiency virus 1 (Yang & Gabuzda, 1999), Borna disease virus (Planz et al., 2001), visna virus (Barber et al., 2002), respiratory syncytial virus (Kong et al., 2004) and coxsackie B3 virus (Luo et al., 2002) have been shown to activate the MAPK pathway upon entry into cells. KSHV entry into cells induces host-cell signalling, which involves phosphatidylinositol 3-kinase, PKCζ and the MAPK/ERK pathway (Naranatt et al., 2003), and several KSHV proteins have been shown to induce angiogenesis and growth deregulation through the activation of multiple host-cell signalling cascades, including the MAPK/ERK and p38 pathways (Munshi et al., 1999; Sodhi et al., 2000; McCormick & Ganem, 2005). In addition, the enhancement of KSHV entry into target cells through regulation of growth factors expression by the Raf/MAPK/ERK pathway has been reported (Akula et al., 2004).

In conclusion, we have demonstrated the activation of the MAPK/ERK pathway as a novel cell-signalling pathway involved in KSHV lytic reactivation following TPA stimulation. Ye et al. (2005) have recently demonstrated an alternative activation pathway of the Rta promoter through the stimulating protein 1 (Sp1) upon n-butyrate treatment of PEL cells, whilst Chen et al. (2001) proposed that demethylation in the lytic promoter region is the essential event necessary for virus lytic replication. On the other hand, overexpression of nuclear factor kappa B (NF-κB) inhibits lytic replication of KSHV, whilst inhibition of NF-κB leads to the expression of KSHV/Rta (Brown et al., 2003). These suggest that a variety of alternative signalling pathways have the potential to induce lytic virus reactivation. Hence, it is likely that a single or combined molecular trigger(s) operate(s) independently or synergistically to stimulate lytic reactivation. Further studies are needed to decipher the physiological conditions that trigger the different pathways.
ACKNOWLEDGEMENTS

We thank Yuan Chang and Patrick Moore for providing the BCP-1 cell line and antibodies for vIL-6. We thank Keiji Ueda for providing antibodies to Rta. This work was supported by the Association for International Cancer Research and by grant no. 6163-1 from the Chief Scientist’s Office of the Ministry of Health, Israel.

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