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MINIREVIEW

Role of Zn^{2+} Ions in Host-Virus Interactions▽

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The involvement of Zn^{2+} ions in the structure of ubiquitous protein zinc fingers gives this metal a particular position in the biology of the cell. Zinc has long been known as a trace element crucial for the proper folding and functioning of numerous proteins in the cell, including enzymes, signal transduction proteins, and transcription factors. However, deeper insights into the exact mechanistic role of zinc in different cellular processes, especially the physiological role of free-Zn^{2+} ions present in the cell, were limited, while the key elements of the cellular system regulating Zn^{2+} trafficking and storage remained unidentified. The discovery of the zinc binding metallothioneins (43) and the subsequent identification of the first zinc transporters in the mid-1990s (53) constituted milestones in this field, leading to a much better understanding of zinc biochemistry. Nevertheless, because of extremely low concentrations of unbound free Zn^{2+} in the cell, progress was long hampered by the lack of highly sensitive and specific Zn^{2+} indicators. Admittedly, it has been known that apart from their role in maintaining the proper zinc finger structure, free-zinc ions somehow affect a variety of signaling pathways, but only very recently has it been demonstrated that free-Zn^{2+} ions can serve not only as modulators of signal transduction but also as classical cellular second messengers (33, 73).

Uncovering these molecular details of zinc homeostasis in the cell has unexpectedly opened new avenues in the field of virology, shedding new light on host-virus interactions. It has long been recognized that Zn^{2+} is an important cofactor not only of cellular proteins but of many viral proteins as well. Recent studies demonstrated that the cellular environment itself, with its extremely small and tightly controlled pool of free zinc, may represent a limiting factor. Viruses rely on the intracellular store of zinc ions and use cellular Zn^{2+} for their newly synthesized proteins. Consequently, the cellular systems controlling zinc balance might constitute a natural protective barrier that limits the accessibility of zinc and thus interferes with virus replication. Indeed, disruption of this barrier, an event that naturally takes place in patients suffering from a rare genetic disease, epidermodysplasia verruciformis (EV), leads to a zinc imbalance in the cell (37) and confers an unusual sensitivity to infections by cutaneous human papillomaviruses (EV-HPVs) (51). Intriguingly, this protective barrier appears to constitute an evolutionarily conserved cellular target for viral proteins, and some viruses have developed an active mechanism that allows them to invade the cellular system managing zinc fluxes in order to maintain the desired free-zinc concentration (37, 49). These findings define a previously unknown type of host-virus interaction, further stressing the importance of cellular Zn^{2+} ions to the virus. However, to what extent this mechanism represents a more general phenomenon in virology remains an open question.

Here, we first present the general basis of the significance of zinc to the virus, and thereafter we discuss the molecular mechanisms of this enigmatic interplay between viruses and the cellular systems that manage Zn^{2+} flux, with particular emphasis on this newly discovered type of host-virus interaction.

ZINC HOMEOSTASIS IN THE CELL

Two distinct, although exchangeable, pools of intracellular zinc can be defined: (i) protein-bound zinc and (ii) the unbound or only loosely bound Zn^{2+}, so-called "free" zinc. Due to the toxicity of zinc to the cell, the vast majority of intracellular zinc belongs to the first pool, and only an extremely small amount of zinc is present as free-zinc ions. The amount of Zn^{2+} might be as much as 10^3 atoms per cell, although the exact concentration and subcellular zinc distribution are still controversial (12). The level of free Zn^{2+} in the cell is tightly controlled, being maintained at a relatively stable level by two complementary mechanisms (Fig. 1). First, an overload of free zinc is rapidly buffered by binding to cellular proteins, in particular to metallothioneins. Metallothioneins constitute a family of cysteine-rich, low-molecular-weight metalloproteins. The main physiological role of metallothioneins in the cell still remains elusive, although it has been demonstrated that metallothioneins protect the cell from metal-induced toxicity. Their expression is induced by different metals, including zinc, and interestingly, they might also be induced in response to viral infections (i.e., coxsackievirus type B and measles virus) (31, 77). The second mechanism involves zinc transporters belonging mainly to two families: ZIP (SLC39) and CDF/ZnT (SLC30) (12, 40, 62). ZIP family members are the proteins that transport zinc from the extracellular space or organellar lumen into the cytoplasm. On the other hand, ZnT family transport-

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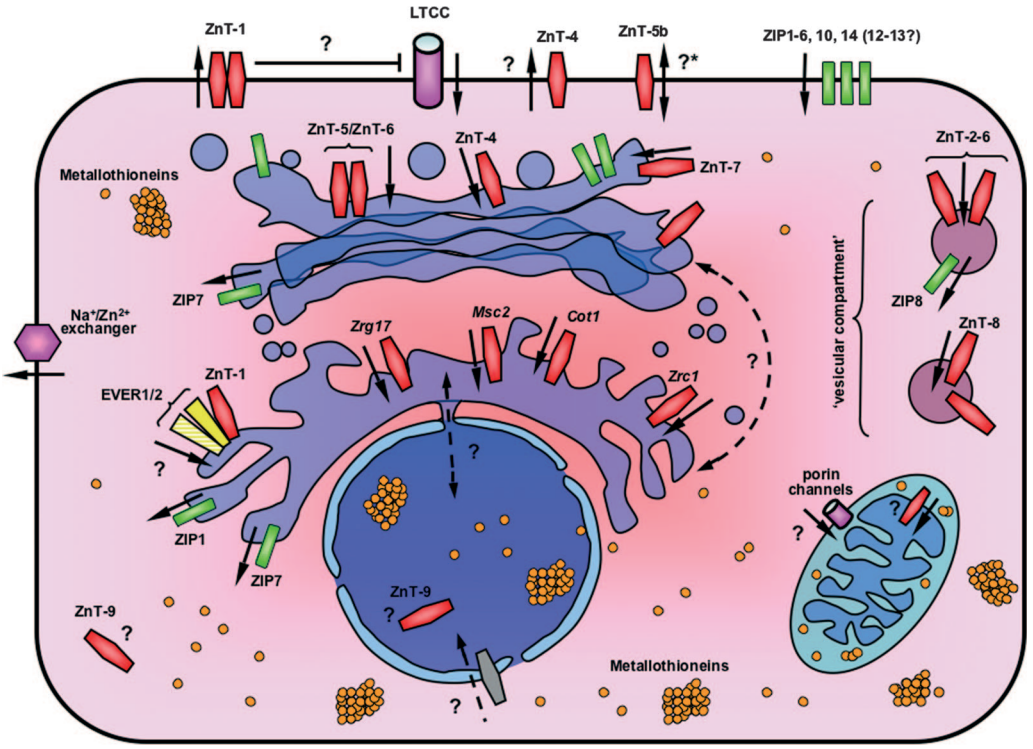

FIG. 1. The system that manages cellular zinc homeostasis. Cellular Zn^{2+} uptake is conferred by ZIP transporters (shown in green), although L-type calcium channels (LTCC) might participate in this process. An excess of unbound cytoplasmic Zn^{2+} is transported outside the cell by ZnT/CDF family members (shown in red), mainly ZnT-1. However, the influence of ZnT-1 on Zn^{2+} transport may also result from indirect suppression of Zn^{2+} influx through L-type calcium channels. The exact role of other ZnTs (ZnT-4 and ZnT-5) and the Na^{+}/Zn^{2+} exchanger in Zn^{2+} efflux is not yet fully clarified and might differ, depending on cellular background. It is noteworthy that the splice variant ZnT-5b (*) constitutes the only known exception, and in contrast to other ZnT members, it might also transport Zn^{2+} into the cytoplasm. The cytoplasmic Zn^{2+} is buffered by binding to cellular proteins, metallothioneins, in particular, or is redistributed among organelles. So far, several proteins transporting Zn^{2+} into the Golgi apparatus have been identified (ZnT-4, ZnT-5, ZnT-6, and ZnT-7). The transfer of Zn^{2+} into the ER in mammalian cells is much less clarified, although the EVER/ZnT-1 complex might be involved in this process. Furthermore, a mutual exchange of Zn^{2+} between the ER and Golgi apparatus through antero- and retrograde vesicular transport has been postulated. Zn^{2+} is transported into the "vesicular compartment" by different sets of ZnT proteins, depending on the tissue context. Zn^{2+} possibly crosses the outer membrane of mitochondria through porin channels; subsequently, it might be bound to the metallothioneins in the intermembrane space and/or further transported to the mitochondrial matrix by not-yet-defined proteins. Zn^{2+} is transported in the reverse direction, from organelles to the cytosol, by ZIP family members, and ZIP1, ZIP7 (ER and Golgi apparatus), and ZIP8 (vesicles) have been implicated in this process. The arrows represent the directions of Zn^{2+} transport (the dashed arrows correspond to the hypothetical routes). The names of nonmammalian ER-residing transporters have been italicized. The "vesicular compartment" comprises different types of cellular vesicles (endosomes, lysosomes, synaptic vesicles, and secretory vesicles) without further distinction, depending on the tissue context.

ers not only exchange Zn^{2+} between different cellular compartments, modulating its local concentration, but also mediate zinc efflux, decreasing the total intracellular zinc content. In addition, the increased intracellular Zn^{2+} level stimulates the expression of zinc transporters through the activation of a zinc-inducible transcription factor named metal-responsive transcription factor 1 (MTF-1) (52, 55), imposing a self-regulating feedback loop. Collectively, metallothioneins and zinc transporters, together with sensing elements like MTF-1, control Zn^{2+} trafficking and storage, maintaining cellular zinc balance. Such a balance is crucial for proper cell functioning, and even small alterations in the free-zinc concentration exert a pleiotropic influence on a variety of cellular processes (i.e., cell signaling and proliferation), while excess zinc is toxic to the cell and induces apoptosis (63).

The significance of precise control of the Zn^{2+} level is further strengthened by recent observations that like Ca^{2+} or cyclic AMP, Zn^{2+} itself serves as a classical intracellular second messenger (73). It has been demonstrated that the activation of cell surface receptor FcεRI leads to the rapid release of Zn^{2+} ions from the internal stores, most likely located in the endoplasmic reticulum (ER), in a calcium influx- and mitogen-activated protein kinase-dependent manner (73). This, in turn, changes the expression of cellular genes and modifies the duration and strength of signaling, possibly by suppressing phosphatase activity. However, in what form zinc is stored in the ER and the molecular mechanism of its release from the ER into the cytoplasm as free ions remain unknown.

VIRUS LIFE CYCLE AND ZINC

There is a growing body of evidence that zinc is important for the invasion of viruses into the cell. Nevertheless, the molecular basis of this interplay between viruses and cellular zinc is still largely unknown. By default, at least two possible, mutually nonexclusive mechanisms exist. First, Zn^{2+} is a well-

known structural cofactor of viral proteins, and second, zinc ions, participating in signal transduction in the cell, could change the activities of different transcription factors and thus the expression patterns of cellular and viral genes.

ZINC AS A STRUCTURAL COFACTOR OF VIRAL PROTEINS

The role of zinc as a protein cofactor is certainly a quite common phenomenon among viruses. Zinc binding proteins have been described for a great variety of both RNA and DNA viruses, such as retroviruses (4), rotaviruses (7, 14), adenoviruses (8), herpesviruses (17), polyomaviruses (24, 70), and papillomaviruses (1, 26), to mention only a few better-studied examples. These viral proteins comprise zinc binding motifs that resemble zinc finger domains found in cellular proteins. Zinc fingers are small, independently folded domains that bind at least one zinc ion. In eukaryotic cells, the most abundant are the proteins with a classical Cys_2His_2 (CCHH) zinc finger motif (36), whereas viral zinc fingers often comprise distinct motifs, different from the classical motif (see below). Both viral and cellular zinc fingers can participate in protein-nucleic acid and protein-protein interactions, and these motifs are usually highly conserved, being critical for protein function.

In discussing the possible interactions between zinc and viral proteins, we focus on the human immunodeficiency virus (HIV), which is one of the best-studied viruses in this context and could serve as a general example. HIV belongs to the family *Retroviridae* and comprises three essential genes common to all retroviruses (*gag*, *pol*, and *env*) and several accessory genes present only in the genomes of some of the *Retroviridae* (e.g., *tat*, *rev*, *nef*, *vpr*, *vpu*, *vif*, and *vpx*), which are thought to adapt the virus to the host cell. Some of the proteins encoded by these genes contain cysteine-rich motifs constituting a putative zinc finger. The role of zinc finger domains has been the most extensively studied role of the structural proteins Gag precursor protein and its derivative, nucleocapsid protein.

The precursor Gag protein encoded by the *gag* gene is involved in binding and packaging viral genomic RNA. Thereafter, the Gag precursor is cleaved by viral protease to yield structural proteins whose numbers differ, depending on the class of retrovirus. At least three structural proteins are encoded by all of them: the matrix protein (MA), capsid protein (CA), and nucleocapsid protein (NC). The HIV NC protein contains two copies of the highly conserved zinc binding motifs, Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC), referred to as "anisotropic" zinc fingers to distinguish them from the canonical eukaryotic zinc fingers with the symmetrical CCHH motif (4). The Gag precursor binds Zn^{2+} ions, and this is necessary for the recognition and encapsidation of viral RNA (4, 25). Moreover, this zinc binding motif seems to be critical for the appropriate cellular trafficking of Gag, and a mutation in the zinc finger excludes the protein from the endosomal compartment, leading to the accumulation of Gag in the cell and interfering with the release of infectious virus (25).

The role of the Zn^{2+} ions bound to the mature NC protein is less clear (4, 67). This issue has raised a large amount of interest, as NC is a crucial protein for both the early and late stages of the virus life cycle and the NC zinc binding motif is mutationally intolerant, suggesting that it might serve as an interesting therapeutical target. Intriguingly, deleting the zinc binding motif in NC results in premature viral DNA synthesis and the generation of noninfectious DNA-containing viral particles (29). Therefore, removal of zinc from the NC zinc finger structure could represent an interesting antiviral strategy, interfering simultaneously with the Gag precursor and mature NC protein function. On top of that, as only very few eukaryotic proteins contain this type of zinc finger, removal of Zn^{2+} from the NC protein by compounds specific for the "anisotropic" finger might constitute a much more selective therapeutical approach than simple zinc chelation. Indeed, it has been demonstrated that several C-nitroso compounds can eject Zn^{2+} from NC zinc fingers without affecting significant cellular zinc binding proteins (58, 59). Interestingly, a similar strategy has been successfully employed to eject zinc from the papillomavirus major oncoprotein E6 (3).

Distinct types of zinc binding domains have been found in several nonstructural HIV proteins—integrase and accessory proteins such as Tat or Vif proteins. Apart from the involvement of viral zinc fingers in interactions with nucleic acids, the zinc binding motifs of integrase, Tat, and Vif are implicated in protein-protein binding. The association of zinc ions with their zinc finger-like structures confers at least three types of interactions: (i) Zn^{2+} allows maintenance of the conformation required for the self-aggregation of viral protein and thus might participate in viral protein multimerization; (ii) the zinc ion could be involved in the generation of metal-linked dimers, binding simultaneously to two distinct molecules of the same protein, thus serving as a sort of bridge; and (iii) zinc might be a critical factor for the interactions between viral proteins and their cellular partners.

A representative of the first type of zinc-dependent protein-protein interactions could, for instance, be retroviral integrase. HIV integrase comprises three distinct domains, the N-terminal, C-terminal, and central core domain. The N-terminal domain contains an invariant zinc finger motif (HHCC), structurally different from both the zinc binding motif of the NC protein and the canonical eukaryotic zinc fingers. The binding of zinc by this domain has been shown to stabilize the structure of the enzyme and to facilitate its multimerization. As the active form of integrase is a multimer, these zinc-mediated interactions also enhance its catalytic activity (38, 39, 46). In contrast, Tat protein might serve as an example of the second and third types of Zn^{2+}-mediated interactions. Tat protein comprises a cysteine-rich region that was shown to bind two Zn^{2+} per one monomer. The unique feature of this structure is that metal serves as a linker between monomers, and binding to two Tat proteins, zinc ions cross-link them (18, 19, 30, 47). A similar mode of Zn^{2+}-mediated cross-linking has recently been proposed for Vif protein (41, 54).

On the other hand, the association of zinc with viral proteins might lead to conformational changes and consequently to the exposure of domains involved in interactions with their cellular partners. In fact, in the cases of both Tat and Vif, zinc was shown to be important for binding by their cellular partners, cyclin T1 (21) and cullin 5 (41, 54), respectively. It has been demonstrated that the binding of Zn^{2+} by Vif protein changes its conformation, leading to its folding predominantly into a β-sheet, which allows it to interact with cullin 5. This association with cullin 5 is crucial for Vif-mediated hijacking of cel-

lular proteasome machinery and the subsequent degradation of the cellular proteins with antiviral activity, in this case APOBEC3G. In the absence of Vif, this host cell cytidine deaminase is incorporated into the virion and targets neosynthesized viral cDNA. Massive deamination leads to hypermutation and finally to degradation, consequently reducing virus infectivity (23). It has been demonstrated that both the chelation of zinc and the deletion of this zinc finger-coding sequence in Vif protein suppress its activity and significantly reduces virus infectivity (41, 54, 72).

ZINC, CELL SIGNALING, AND THE VIRUS

The role of zinc signaling in the virus life cycle has been much less studied. Clearly, zinc ions change the global phosphorylation of cellular proteins, which obviously might affect the virus life cycle. It has been proposed that phosphatases (PTPs) constitute a cellular target for zinc. Almost all of the cellular PTPs contain a cysteine residue in the active site, which exists in a thiolate form. Zn^{2+} can react with these PTP cysteines and thus inhibit their function (2, 27). Moreover, an excess of zinc ions can induce the generation of radical oxygen species (20), which also react with PTP cysteines, suppressing their activity (2). In addition, radical oxygen species themselves may release zinc from proteins, in particular from metallothioneins, further increasing the pool of free-zinc ions. Thus, through an increase in the phosphorylation of cellular proteins, zinc might activate some signaling pathways, and in turn, it could alter the activities of transcription factors, changing the expression of target genes relevant to the virus.

Indeed, zinc was shown to activate, among others, the phosphoinositide 3-kinase/Akt and extracellular signal-regulated kinase/Jun N-terminal protein kinase (JNK) (2, 13, 32, 63) pathways, which could presumably affect the life cycles of numerous viruses. For instance, the zinc-mediated activation of JNK, a cellular kinase involved in the phosphorylation and activation of c-Jun, a constituent of AP-1 transcription factors, stimulates AP-1 transcriptional activity (32; our unpublished data). This might have an impact on the virus in at least two different ways. First, directly, as AP-1 is a cellular transcription factor, which is important, or even critical in some instances, as in the case of papillomaviruses, for the expression of viral genes (35, 75). On the other hand, through the modulation of AP-1 activity, free zinc changes the expression levels of multiple cellular genes, among which are those relevant to the virus, e.g., constituents of the natural antiviral response (for example, cytokines).

However, it needs to be emphasized that many of these studies on zinc signaling have been performed under conditions in which cells were exposed to supraphysiological concentrations of zinc and could reflect a physiological situation to only a limited extent. Nevertheless, recent investigations demonstrated that Zn^{2+} also inhibits phosphatase activity in the range of physiological concentrations (73). It has been shown that physiological oscillations of the intracellular pool of free zinc affect protein phosphorylation and alter the expression levels of cellular genes. A burning question is to what extent these physiological oscillations of free zinc in the cell, within the range of very low concentrations, could be important for the virus.

FREE-ZN^{2+} CONCENTRATIONS AND THE OPTIMAL ENVIRONMENT FOR THE VIRUS

Even though it seems obvious that deviations in zinc concentrations from the given, optimal level desired by the virus could exert an effect on its life cycle, a distinct issue is whether the physiological concentrations of cellular free Zn^{2+} fit the demands of the virus. This is still a matter of debate, and there are a few fundamental questions related to this issue. (i) What is the viral protein requirement for Zn^{2+} as a cofactor, and is the physiological concentration of free zinc in the infected cell optimal for the function of these proteins? (ii) How are Zn^{2+} ions delivered to the newly synthesized viral proteins? (iii) How does the cellular system controlling zinc homeostasis in the cell respond to the increased global demands for Zn^{2+} ions in the infected cell? (iv) What might be the consequences of such a virus-induced deficit of free zinc on cell function and the virus life cycle? (v) Is the steady-state level of cellular free zinc optimal for the virus in terms of the activities of cellular transcription factors, or is an increased free-Zn^{2+} pool needed for efficient viral gene expression and replication? (vi) Finally, could viruses actively regulate the cellular systems controlling zinc homeostasis, changing the "settings" of the system in order to adjust it to the optimal level for the virus's demands?

Even though many of these questions remain unanswered, recent studies have provided some breakthroughs. As a relative deficit of free zinc could negatively affect the structure and function of viral proteins, one might speculate that maintaining a higher concentration of free zinc in the cell could generally constitute an advantage for the virus. It seems that at least for some viruses, the physiological level of free zinc is suboptimal and that changing the zinc balance is beneficial for the virus. Such a zinc imbalance could be conferred by (i) an inherited defect in the cellular systems managing zinc fluxes or (ii) by some viral proteins taking control over Zn^{2+} trafficking in the cell in order to keep a higher concentration of free zinc. In fact, recent studies suggest that both situations take place. Intriguingly, it has recently been found that patients suffering from EV (OMIM accession number 226400), a rare genodermatosis characterized by unusual sensitivity to papillomavirus infections, display a dysfunction in one of the zinc-transporting complexes. On the other hand, we have found that both the E6 and E7 proteins of HPV are able to interact with metallothioneins (our unpublished data), presumably to release free-zinc ions. In this context, metallothioneins could serve as the proteins that deliver Zn^{2+} directly to these viral proteins (or simply release Zn^{2+} upon viral protein binding, increasing the local concentration of zinc ions in the closest neighborhood of E6 and E7), thus ensuring their proper conformation and function. There is also speculation that the interaction between viral proteins and metallothioneins favors the release of free zinc, which activates signaling pathways (13), leading to the synthesis of the transcription factors needed for viral gene expression.

EV AS A NATURAL MODEL OF ZINC DYSREGULATION IN HPV INFECTIONS

EV is a rare human skin disease (42, 50, 51) in which the sole identified primary defect is an abnormal, highly selective sus-

ceptibility to a specific group of ubiquitous cutaneous HPVs, the so-called EV-HPVs, but not to other closely related HPVs, including genital HPVs (15, 51). HPVs are small, nonenveloped DNA viruses which are classified into five different phylogenetic genera (10). Genital HPVs are classified into the *Alphapapillomavirus* genus, whereas EV-HPVs belong to the distinct *Betapapillomavirus* genus. The HPV genome is circular and contains open reading frames encoding late proteins (L1 and L2), which are structural capsid proteins, and early proteins (E1, E2, E4, E5, E6, and E7), which coordinate replication and expression of the viral genome and are involved in the adaptation of the functions of the cell to the requirements of the virus (15). Papillomaviruses infect the keratinocytes of humans and a wide range of animal species and induce a large variety of lesions, from benign skin warts to invasive cancers (15, 51, 61). Clinical HPV genital infections are the most common sexually transmitted diseases in the world. The mechanisms of HPV-induced malignant transformation have been extensively studied, and two major viral oncoproteins, E6 and E7, which are zinc binding proteins (1, 26), have been identified and characterized in detail (78).

In contrast to genital HPVs, EV-HPVs, although ubiquitous, are usually harmless in the general population, causing widespread, asymptomatic infections. However, in patients suffering from EV, oncogenic HPV5 and HPV8 replicate very efficiently and reveal their full transforming potential, inducing multiple nonmelanoma skin cancers (51), which strongly suggests the existence of a natural antiviral barrier in the host cells. The majority of EV cases are caused by mutations in one of the two adjacent genes *EVER1* or *EVER2* (51, 56, 57). The *EVER1* and *EVER2* genes are evolutionarily conserved and encode proteins belonging to the transmembrane channel-like (TMC) family (34). Among the eight *TMC* genes, *EVER1* and *EVER2* correspond to *TMC6* and *TMC8*, respectively. In keratinocytes, EVER proteins are located mainly in the ER (37, 57), and it has been speculated that like other TMC members, these two proteins may play a role in ion transport or the modulation of signal transduction.

PHYSIOLOGICAL ROLE OF EVER PROTEINS

We have recently reported that in human keratinocytes, EVER proteins form a complex in the ER with ZnT-1 and participate in maintaining cellular zinc homeostasis. The ZnT-1/EVER complex is involved in the regulation of Zn^{2+} trafficking in the cell and seems to control Zn^{2+} influx into the nucleus (37). Since the molecular mechanism of Zn^{2+} transfer across the nuclear membrane is itself poorly understood, the mode of action of EVER proteins remains speculative. It has been demonstrated that the ER might constitute an important organelle where zinc is stored and from where, upon the triggering of the cell surface receptor, a reservoir of Zn^{2+} can be rapidly released into the cytoplasm and subsequently passed to the nucleus, in what has been designated a "zinc wave" (73). Most likely, the ER-residing ZnT-1/EVER complex could be involved in the transfer of Zn^{2+} in the reverse direction, from the cytoplasm into the ER lumen, interfering with the spreading of the zinc wave and the function of Zn^{2+} as a second messenger. Thus, by decreasing the free-zinc concentration in the cytoplasm, the ZnT-1/EVER complex might indirectly alter Zn^{2+} transport from the cytoplasm to the nucleus. The exact role of EVER proteins within the ZnT-1/EVER complex remains to be determined, and EVERs might either (i) only modulate the activity of ZnT-1, a known zinc transporter, or (ii) serve as Zn^{2+} transporters themselves, based on the homology of the structure with those of other TMC members.

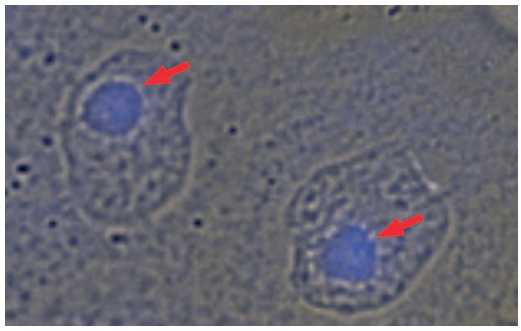

FIG. 2. Free-zinc ions in keratinocytes. Keratinocytes with a mutated *EVER* gene (*EVER2*$^{-/-}$) were loaded with Zinquin, a fluorescent indicator specific for free zinc; subsequently, the cells were incubated for 15 min in 100 μM zinc. A strong fluorescence, corresponding to places with a high concentration of free zinc, is visible mainly in the nucleoli (indicated by red arrows).

Regardless of the exact mechanism, a mutation in one of the *EVER* genes and the lack of the corresponding protein disrupt the function of the whole ZnT-1/EVER complex. When human keratinocytes are loaded with a fluorescent indicator specific for free zinc, a bright fluorescence appears in the nucleoli and is further augmented when the cells are exposed to a high concentration of zinc (37) (Fig. 2). However, it has been shown that the fluorescence intensity of the nucleoli is significantly higher in *EVER2*$^{-/-}$ cells than in *EVER2*$^{+/+}$ cells, demonstrating an abnormal distribution of intracellular free zinc in EVER-deficient cells that could be interpreted as a retention of zinc within the nucleus (37). Notably, this redistribution of the free-zinc pool is accompanied by enhanced transcriptional activity of MTF-1 (37). Interestingly, apart from an effect on the transcription factor that is directly zinc inducible, ZnT-1 and EVER proteins have been shown to suppress the activity of c-Jun, an important constituent of the AP-1 transcription factor. This effect might be mediated by the inhibition of the zinc-induced activation of JNK (13, 32) but does not necessarily involve glycogen synthase kinase 3β (37; our unpublished data), another c-Jun kinase that in contrast to JNK, suppresses the activity of c-Jun upon phosphorylation (5).

It needs to be emphasized that the influence of the EVER/ZnT-1 complex on the status of c-Jun phosphorylation, probably through changes in the level of Zn^{2+}, certainly reflects much broader alterations in cell signaling and transcription factor activity (37; our unpublished data). Interestingly, even a transient increase in the intracellular concentration of Zn^{2+}, which remains within the physiological range, confers a general suppression of cellular phosphatase activity and increases the phosphorylation of multiple cellular proteins (27, 73). Thus, cellular phosphatases constitute a presumptive target for the ZnT-1/EVER complex, and their disinhibition in response to the downregulation of free zinc by the ZnT-1/EVER complex

might in turn globally affect cell signaling and modulate the activity of numerous transcription factors.

ROLE OF EVER PROTEINS IN INFECTED KERATINOCYTES

Although widely distributed in the general population, EV-HPVs are usually detected solely by a highly sensitive nested-PCR approach; in EV patients, the number of copies of viral DNA in keratinocytes is much higher. Thus, the existence of a natural protective mechanism in the host cells has to be assumed. Presumably, in EV patients this selective barrier might be disrupted or compromised as a consequence of *EVER* mutations (56, 57), imposing a host restriction on EV-HPV. These mutations that lead to an increase in cellular free zinc activate JNK-related pathways and induce the transcriptional activity of AP-1 (13, 32, 37). This might constitute an important promoting factor for the HPV life cycle, since AP-1 transcription factors are essential for the expression of the HPV genome (35, 75) and, notably, for virus-induced carcinogenesis (75), at least as far as alphapapillomaviruses are concerned. Thus, a massive expression of viral genes and efficient virus replication are induced in EVER-deficient keratinocytes, eventually conferring malignant transformation. However, a distinct HPV-independent mechanism of skin carcinogenesis in EV patients cannot be completely ruled out. It has previously been shown that forced overexpression of AP-1 itself can increase the proliferation rate and participate in cell transformation. In accordance with these findings, we have observed that the *EVER2* mutation is also associated with the enhanced growth potential of keratinocytes in the absence of HPV (37).

Despite the recent advances in understanding of the role of EVER proteins in cell physiology, the intriguing selectivity of the EVER-based molecular barrier and the lack of protection against genital HPVs remain a mystery. Two possible scenarios can be proposed: (i) the EVER-based barrier could indeed be highly selective toward betapapillomaviruses (β-HPV), blocking some cellular processes that are crucial solely for these papillomaviruses, not for genital HPVs; and (ii) although the barrier controls processes equally important for the life cycles of both alpha- and β-HPVs, genital HPVs might have developed an evolutionary mechanism that enables them to bypass the EVER-based barrier. Recent observations seem to favor the latter possibility (37, 49). As the genomes of genital HPVs contain open reading frame E5, in contrast to those of EV-HPVs, the E5 protein emerged as the first presumable permissive factor that might allow genital HPVs to break the EVER barrier.

HPV AS AN ACTIVE ELEMENT IN THE MANAGEMENT OF CELLULAR ZINC FLUXES

HPV E5 is a highly hydrophobic transmembrane protein, localized in the cell predominantly in the ER, the Golgi apparatus, and nuclear membranes (69). The exact molecular mechanism of HPV E5 action is not fully elucidated. Initially, it was noted that the bovine papillomavirus (BPV) E5 protein can augment the transforming activity of the epithelial growth factor receptor (EGFR) (44), and thereafter it was postulated that HPV E5 might act similarly. Indeed, HPV type 16 (HPV16) E5 was shown to enhance epithelial growth factor (EGF)-triggered signaling, possibly due to the E5-mediated inhibition of EGFR cellular recycling and, in consequence, to the retention of EGFR at the cellular membrane (66, 69). Similarly to BPV E5, the HPV16 E5 protein binds to the 16-kDa subunit of the proton pump [(H^+)-ATPase] (6), thereby possibly inhibiting vesicular acidification and preventing EGFR degradation. However, the binding of HPV E5 to (H^+)-ATPase seems to be insufficient to confer HPV E5-mediated enhancement of EGF signaling (60), and HPV E5 can directly block the proteasomal degradation of EGFR by inhibiting the interaction between EGFR and ubiquitin ligase (76). Nevertheless, even though the possibility of a link between HPV E5 and EGF-related signaling has been raised by multiple studies from different laboratories and is generally well accepted, it must be emphasized that HPV E5 displays little or no homology with BPV E5 and that some of its biological effects are clearly EGFR independent (16).

A new insight into the molecular mechanism of action of the HPV E5 protein came from recent studies on the function of the EVER and ZnT-1 proteins. First, it was reported that HPV E5 protein interacts with ZnT-1, suggesting a possible involvement of HPV E5 in the disruption of the system controlling cellular zinc homeostasis (37, 49). Interestingly, a similar interaction with ZnT-1 was found in the case of cottontail rabbit papillomavirus E8 protein (49), a transmembrane oncoprotein structurally and functionally related to E5 (28). These findings further suggest that zinc homeostasis in the cell might represent a common cellular target for different papillomaviruses. Subsequently, we have shown that HPV E5 interacts with EVER proteins and colocalizes with the EVER/ZnT-1 complex in keratinocytes (37). More importantly, HPV16 E5 has been shown to influence the cellular system controlling zinc balance, leading to an increase in the concentration of intracellular free Zn^{2+} in keratinocytes, as measured by the activity of the zinc-inducible transcription factor MTF-1. In addition, HPV E5 interfered with EVER2-mediated inhibition of EGF-induced c-Jun activity in human keratinocytes (37). Thus, HPV E5 exerts an influence strikingly similar to that of EVER deficiency, functionally mimicking the effect of *EVER* mutations in EV patients. As zinc inhibits cellular phosphatases and was shown to increase the phosphorylation of EGFR as well (68), the influence of HPV E5 on the cellular system controlling zinc homeostasis might integrate the previously published results. On the one hand, it could explain the well-documented effect of HPV E5 on EGF-triggered signaling, and on the other hand, as changes in free zinc are likely to exert a rather global effect in the cell, it could also explain some of the EGFR-independent HPV E5 activities.

Although HPV E5 protein is not an indispensable factor for the virus, it does ensure proliferative competence in more-differentiated cells, and it augments viral genome amplification and regulates the expression of other viral genes (16, 22). Therefore, E5 is thought to be involved in the coordination of the HPV life cycle and is important mainly during the productive stages (11). We propose that EVER deficiency could play a similar role for EV-HPVs. Indeed, it has been demonstrated that a mutation in *EVER2* in keratinocytes compensates for the lack of the functional E5 protein in β-HPVs, explaining the host restriction in the case of EV (37). On the other hand,

the significance of the interaction between E5 protein and the EVER complex and modifications of cellular zinc balance could have some important implications not only for the virus but also for the host cell itself. Although HPV E5 does not display full transforming capacity, it can potentiate the oncogenic activity of E6/E7 proteins (65), which even though constituting more of a "side effect" for the virus, is important to fully understand the pathology of HPV-related cancers. Using a transgenic murine model, it has been demonstrated that although the HPV E5 protein is not a major factor involved in the initiation step of papilloma formation, it does contribute to the promotion and progression stages of carcinogenesis (45). Moreover, in this model, HPV E5 conferred an endophytic and highly penetrant type of cancer growth. It is tempting to speculate that this biological effect of HPV E5 action could be related to its influence on EVER/ZnT-1 function and in turn on cellular zinc homeostasis. Remarkably, a similar, highly penetrating and invasive mode of tumor growth with a propensity to metastasize in many cases has been observed in EV patients (9). We hypothesize that breaking the EVER barrier, regardless of whether it is due to a mutation in either of the *EVER* genes or to the effect of E5, could constitute a tumor-promoting factor, which changes the natural evolution of the developing cancer.

All these basic findings point to the deregulation of the cellular system controlling zinc homeostasis as a crucial step in the HPV life cycle, both for alphapapillomaviruses and β-HPVs (genital HPVs and EV-HPVs). However, two distinct molecular mechanisms could be involved: E5-mediated suppression of EVER/ZnT-1 complex activity for genital HPVs and an *EVER* mutation for β-HPVs. One might pose the question, why did EV-HPVs evolve to be devoid of an apparently important mechanism facilitating their replication? The high viral load found in some EV patients suggests that inhibition of the EVER complex might indeed be helpful for β-HPV genome amplification. This can be understood at least in the context of oncogenic EV-HPVs when one considers the clinical phenotype of EV patients. In contrast to the oncogenic genital HPVs like HPV16 or HPV18, in the case of which the majority of infections do not confer a clinically relevant malignancy, β-HPVs consistently lead to cancerous transformation, as only they infect EVER-deficient cells. Possibly, important qualitative differences between the alphapapillomaviruses and β-HPVs in the set of early genes other than E5 could explain this striking contrast observed in cells with disrupted EVER complexes. Thus, taking into account the whole repertoire of viral genes, the existence of a mechanism that disrupts zinc balance could dramatically shorten the life expectancy of the infected host and eventually might not necessarily constitute an advantage for EV-HPVs. Therefore, β-HPVs appear to be viruses that are remarkably well adapted to their hosts and do not need to deregulate the cellular zinc balance in order to efficiently spread in the human population. On the other hand, genital HPVs represent the first known examples of the virus, which evolved to actively control the cellular zinc balance, which could be beneficial for the virus life cycle and, in most cases, will not result in fatal consequences for the host.

CONCLUDING REMARKS

A major advance in the understanding of host-microbe interactions often originates at the frontiers of distinct fields. A better understanding of the molecular mechanisms controlling cellular zinc homeostasis has enabled more mechanistic studies on the functions of the proteins encoded by the *EVER* genes, shedding new light on the molecular foundation of EV. In turn, this important insight into the pathogenesis of EV, an extremely rare genetic disease, brought new clues to the biology of the widespread oncogenic papillomaviruses. Finally, the integration of all of these findings from seemingly distinct fields led to the discovery of a previously unknown type of host-virus interaction, showing that the mechanisms controlling cellular Zn^{2+} trafficking constitute natural antiviral barriers. This, together with the previously gathered broad knowledge of the role of Zn^{2+} as a cofactor of viral proteins, suggests that the accessibility of zinc ions in infected cells could be a potentially limiting factor in the virus life cycle.

It remains an intriguing question whether this type of interplay between viruses and cellular zinc might be a more-universal phenomenon, conserved among distinct groups of viruses. Interestingly, an increase in c-Jun phosphorylation and the constitutive activation of AP-1 have been reported as important viral strategies in the case of polyomaviruses, for example (64), and inhibition of AP-1 activity can interfere with the transforming potential of viral proteins (71). In this context, it is possible that the deregulation of cellular zinc homeostasis by the virus, which leads to persistent activation of AP-1, might simply constitute yet another evolutionarily selected viral strategy dedicated to maintaining high levels of activity of the desired transcription factors.

Moreover, the recent findings have some general implications for the specific field of papillomavirus-associated diseases, including EV itself. Specifically, the novel insight into the pathogenesis of HPV infections defines new directions in the search for future therapeutic strategies. One such strategy might be an attempt to increase *EVER* expression in order to prevent cellular zinc imbalance. Overexpression of ZnT-1 and EVER proteins has already been shown to suppress the activities of AP-1 and MTF-1 (37). More importantly, in transfection experiments, an *EVER2* mutation leading to the lack of the functional EVER2 protein can be compensated by overexpression of the EVER1 or ZnT-1 protein (our unpublished data). All EV patients carry a wild-type copy of one of the *EVER* genes (either the *EVER2* wild-type for *EVER1*$^{-/-}$ individuals or the *EVER1* wild-type for *EVER2*$^{-/-}$ individuals) in their genomes, which implies that the "pharmacological" enhancement of expression of the remaining wild-type gene might in general constitute an interesting therapeutic approach for these patients. Conceptually similar approaches in which a missing protein is replaced by the overexpression of the gene encoding the other protein have already been proposed in different fields, for muscular dystrophies, for example (48). However, currently nothing is known about the physiological mechanisms controlling the expression of the *EVER* genes, and a detailed analysis on that subject is more than desirable. It would also be of interest to verify whether such an increased expression of EVER proteins could prevent the HPV E5-induced increase in cellular free zinc in infected keratino-

cytes, thus conferring a rather generally augmented protection against papillomaviruses. If so, it could open completely new avenues for preventive and/or therapeutic strategies in the field of genital HPVs as well. On the other hand, as we postulated that the clinical phenotype in EV patients could be causatively associated with the increased activity of JNK and c-Jun, pharmacological suppression of this pathway might also represent an attractive approach for these patients. The successful therapeutical employment of a JNK inhibitor (SP600125) in vivo has recently been reported (74).

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