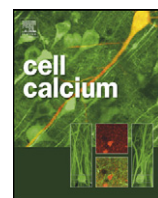Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Review

Viral calciomics: Interplays between Ca^{2+} and virus

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ARTICLE INFO

ABSTRACT

Ca^{2+} is one of the most universal and versatile signaling molecules and is involved in almost every aspect of cellular processes. Viruses are adept at utilizing the universal Ca^{2+} signal to create a tailored cellular environment that meets their own demands. This review summarizes most of the known mechanisms by which viruses perturb Ca^{2+} homeostasis and utilize Ca^{2+} and cellular Ca^{2+}-binding proteins to their benefit in their replication cycles. Ca^{2+} plays important roles in virion structure formation, virus entry, viral gene expression, posttranslational processing of viral proteins and virion maturation and release. As part of the review, we introduce an algorithm to identify linear "EF-hand" Ca^{2+}-binding motifs which resulted in the prediction of a total of 93 previously unrecognized Ca^{2+}-binding motifs in virus proteins. Many of these proteins are nonstructural proteins, a class of proteins among which Ca^{2+} interactions had not been formerly appreciated. The presence of linear Ca^{2+}-binding motifs in viral proteins enlarges the spectrum of Ca^{2+}–virus interplay and expands the total scenario of viral calciomics.

1. Introduction

Ca^{2+}, chosen by nature through evolution as one of the most universal and versatile carriers of signals, acts as a second messenger inside mammalian cells to regulate almost all aspects of cellular processes, beginning with triggering life during fertilization and ending with the perish of cells via programmed cell death or apoptosis [1,2]. The versatility in speed, amplitude and spatial-temporal patterning of Ca^{2+} signaling allows for control of cellular processes by modulating the activity of a repertoire of signaling components, including receptors, ion channels, pumps, exchangers, Ca^{2+} buffers, Ca^{2+} effectors, Ca^{2+}-sensitive enzymes and transcription factors, in a number of cell compartments [3].

Having evolved to be adept at hijacking the host cell machinery, viruses appropriate or interrupt both Ca^{2+} signaling pathways and other Ca^{2+}-dependent processes to achieve their own ends (Fig. 1). Ca^{2+} has been found to play a role in almost every step in virus replication cycles. The interplay between viruses and Ca^{2+} in the infected cell falls in general into three major categories: (1) viral proteins directly or indirectly disturb Ca^{2+} homeostasis by altering membrane permeability and/or manipulating key components of the Ca^{2+}-signaling apparatus; (2) viral proteins directly bind to Ca^{2+}

for structural integrity or functionality; and (3) critical virus–host interactions depend on cellular Ca^{2+}-regulated proteins or pathways. This review, which aims to stimulate future studies on the roles of Ca^{2+} in virus infection, is focused on such interplay by highlighting recent discoveries in the biological roles of Ca^{2+} in virus infection.

2. Effects of viral infections on Ca^{2+} homeostasis

The concentration of Ca^{2+} outside the cell reaches as high as mM, whereas the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{CYT}$) at resting state is maintained at $\sim$100 nM and the endoplasmic reticulum (ER) Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) at several hundred μM. Thus, cells have to maintain a >10,000-fold gradient across the plasma membrane, and meanwhile, to achieve intracellular Ca^{2+} homeostasis to avoid acute, gigantic fluctuation of Ca^{2+} [3]. A specific spatiotemporal pattern of cytosolic Ca^{2+} signaling is made possible by Ca^{2+} from two major sources: the internal Ca^{2+} store (mainly ER or sarcoplasmic reticulum) and the extracellular medium (Fig. 1). The entry of Ca^{2+} across the plasma membrane, usually triggered by stimuli that include membrane depolarization, mechanical stretch, external agonists, depletion of internal stores and intracellular messengers, is mediated by specific receptors and Ca^{2+} channels. The mobilization of Ca^{2+} from the internal stores in response to Ca^{2+} itself or intracellular messenger, is primarily mediated by the IP$_3$ receptors (IP$_3$R) [4,5] and the ryanodine receptors (RyR) [6]. Several distinct mechanisms (the plasma membrane Ca^{2+}-ATPase (PMCA),
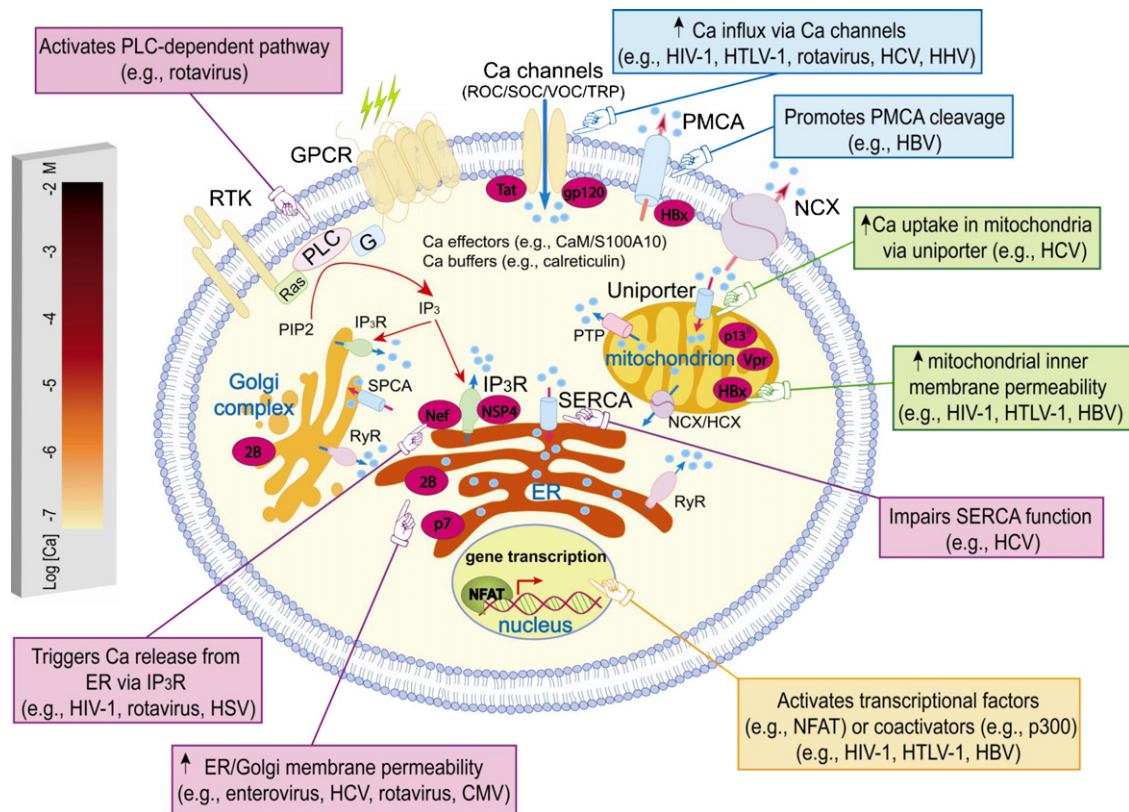
Fig. 1. The choreography of Ca²⁺ signaling and examples of virus-induced perturbations on Ca²⁺ homeostasis. Upon extracellular stimulation, the free cytosolic Ca²⁺ ([Ca²⁺]$_{CYT}$) rapidly increases by the entry of extracellular Ca²⁺ across the plasma membrane via Ca²⁺ channels, such as voltage-operated channels (VOC), receptor-operated channels (ROC), transient receptor potential ion-channel (TRP) and store-operated channels (SOC), or by the release of Ca²⁺ from internal stores (e.g., endoplasmic reticulum (ER), Golgi complex, and lysosomes) through inositol-1,4,5-triphosphate receptors (IP₃R) and ryanodine receptors (RyR) due to activation of membrane receptors (G protein coupled receptor [GPCR] and receptor tyrosine kinase [RTK]) and the subsequent synthesis of IP₃. At the resting state, [Ca²⁺]$_{CYT}$ is maintained at submicromolar range by extruding Ca²⁺ outside of the plasma membrane via plasma membrane Ca²⁺-ATPase (PMCA) and Na²⁺/Ca²⁺ exchanger (NCX), or by pumping Ca²⁺ back into internal stores through sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) or secretory pathway Ca²⁺-ATPase (SPCA). In mitochondria, Ca²⁺ can easily pass through outer mitochondrial membrane pores and cross the inner mitochondrial membrane through the membrane-embedded Ca²⁺ uniporter. Ca²⁺ exits mitochondria through the opening of a non-selective high-conductance channel permeability transition pore (PTP) in the inner mitochondrial membrane and the Na⁺/Ca²⁺ exchanger (NCX). The Ca²⁺ signals are delivered by affecting the activity of Ca²⁺ buffers (e.g., calreticulin), Ca²⁺ effectors (e.g., CaM and S100A10) and Ca²⁺-regulated enzymes. The signals can also have "long-term" effects by modulating the activity of transcriptional factors including nuclear factor of activated T cells (NFAT) or Ca²⁺-dependent transcriptional cotransactivators (e.g., p300). Viral proteins are capable of perturbing the intracellular Ca²⁺ homeostasis by (1) modulating Ca²⁺ pumps and/or channels on the plasma membrane (e.g., Tat and gp120 of HIV-1, HBx of HBV; blue boxes); (2) triggering Ca²⁺ release from internal stores via IP₃R (e.g., NSP4 of rotavirus and Nef of HIV; purple boxes) or altering membrane permeability and pump activity of internal stores (e.g., 2B of coxsackievirus, p7 of HCV, and core protein of HCV; purple boxes,); (3) disrupting mitochondrial membrane permeabilization or potential (e.g., Vpr of HIV-1, p13^{II} of HTLV-1, core protein of HCV and HBx of HBV; lemon boxes); (4) activating Ca²⁺-responsive transcriptional factors or coactivators, such as p300 (e.g., p12^{I} of HTLV-1 and Vpr of HIV-1; yellow box) in the nucleus. Moreover, a variety of viral proteins interact with important cellular CaBPs, such as CaM, S100A10 and calreticulin, to remodel the Ca²⁺ signaling network. The gradient color bar on the left indicates free Ca²⁺ concentration in subcellular compartments. Calcium ions are shown as cyan dots.

the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA), the secretory pathway Ca²⁺-ATPase (SPCA), the Na⁺/Ca²⁺ exchanger (NCX), and the mitochondrial uniporter) are responsible for sequestering Ca²⁺ from the cytosol by transporting Ca²⁺ either to external medium or into different cellular compartments [3,7–9].

The Ca²⁺ signaling system requires the exquisite choreography of the Ca²⁺-signaling toolkit (Ca²⁺ "signalsomes") and undergoes constant remodeling to meet the specific spatial-temporal requirements in a flexible yet precise manner. This flexibility, on the one hand, provides sufficient opportunities for the host cell to adjust to the virus infection. On the other hand, viruses are adept at utilizing the universal Ca²⁺ signal to create a tailored cellular environment that meets their own demands. The development of a variety of cell-permeable Ca²⁺ indicators (such as the acetoxymethyl ester forms of Fura-2, Fluo-4 and Indo-1) and genetically encoded fluorescent indicators (such as cameleon, pericam and aequorin) [10,11] provides solid foundation for the quantitative measurements of cellular Ca²⁺ signal changes in response to virus infection or the expression of viral proteins. In general, virus infection leads to an increase of [Ca²⁺]$_{CYT}$ due to increased plasma membrane permeability and

altered membrane permeability of internal Ca²⁺ stores, such as ER and mitochondria. This observation triggers one immediate question: why do most virus infections elicit an alteration in cytoplasmic Ca²⁺ concentration, but not other metal ions (e.g., Mg²⁺, K⁺, Na⁺)? Answers lie in the unique physiochemical and physiological nature of Ca²⁺. First of all, Ca²⁺ has been chosen by nature through evolution as a versatile second messenger to regulate almost all aspects of cellular processes. As intimate intracellular parasites, viruses are naturally expected to hijack important Ca²⁺ signaling networks to achieve their coexistence with host cells. Second, compared to the much narrower dynamic range of monovalent K⁺ and Na⁺ (<100-fold) and Mg²⁺ (<10-fold) in mammalian cells, a >10,000-fold gradient of Ca²⁺ is maintained across the plasma membrane. This feature gives the virus ample space to manipulate the gradient between membranous compartments to transduce various signals with ease. Last but not least, acute changes of K⁺ and Na⁺ at the mM range would lead to osmotic shock and abrupt disruption of membrane potential. In contrast, the change of Ca²⁺ at nM or μM levels is more subtle and tolerable, thereby circumventing these detrimental effects.
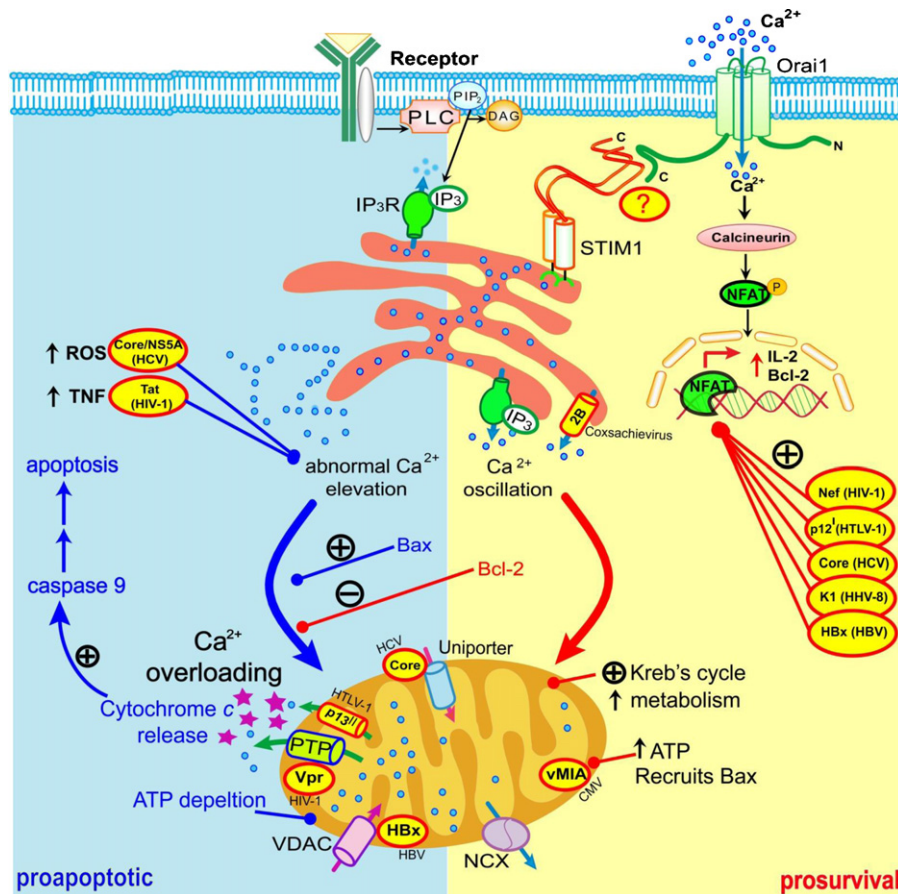
Fig. 2. Virus infection selectively perturbs pro-apoptotic and pro-survival ER-mitochondria Ca^{2+} signaling. Upon engagement of receptors, the production of IP_3 leads to the activation of IP_3R and release of Ca^{2+} from internal store. The decrease of ER Ca^{2+} concentration is subsequently sensed by the EF-hand-containing ER Ca^{2+} sensor STIM1, which in turn activates the CRAC channels through its direct interaction with the pore-forming subunit Orai1, followed by a second phase of intracellular Ca^{2+} elevation (store-operated Ca^{2+} entry; high frequency cytosolic Ca^{2+} oscillation with low amplitude) and further activation of downstream effectors, including calcineurin, NFAT and NFAT-dependent gene expression (e.g., Bcl-2 and IL-2) [53,171]. Overexpression of anti-apoptotic Bcl-2 family members (e.g., Bcl-2) inhibits Ca^{2+} release from ER and thus exerts anti-apoptotic effects to promote survival. NFAT can be activated by viral proteins (e.g., HIV-1 Nef [12], HTLV-1 p12[I] [13,14], HCV core protein [15,16], and HHV-8 K1 [17], HBV HBx [62,63]; red ovals) to enhance virus replication or to establish persistent infection. It is of great interest to test whether viral proteins (red oval with question mark) can directly target SOC components (STIM1 and Orai1) to affect NFAT activation and ER Ca^{2+} store refilling. A decrease in $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_{Golgi}$ (for example, induced by viroporin, 2B of Coxsakievirus; red cylinder highlighted in yellow) may inhibit protein trafficking pathways and cause intracellular accumulation of ER or Golgi-derived secretory vesicles, at which viral RNA replication takes place [20,21]. Ca^{2+} released from ER can be readily taken up by mitochondria at locations in close proximity to the ER extruding pores (so-called ER-mitochondria Ca^{2+} flux). Abnormal exodus of ER Ca^{2+} could result in Ca^{2+} overloading in mitochondria. Mitochondrial Ca^{2+} uptake is mediated by the mitochondrial voltage-dependent anion channel (VDAC) across outer mitochondrial membrane (OMM) and the Ca^{2+} uniporter of inner mitochondrial membrane (IMM). Ca^{2+} exits mitochondria through the opening of a non-selective high-conductance channel permeability transition pore (PTP) in IMM and the Na^+/Ca^{2+} exchanger (NCX) [85,86]. During virus infection, viral proteins may readily target mitochondria and exert either pro-apoptotic or anti-apoptotic action by altering mitochondrial Ca^{2+} levels. A modest increase of ER-mitochondria Ca^{2+} flux may boost ATP production by activating Ca^{2+}-dependent Kreb's cycle dehydrogenases, thereby meeting higher energy demand due to active viral replication (e.g., HCV [18] and CMV [19]). However, Ca^{2+} overloading in mitochondria exerts pro-apoptotic effects. It activates the opening of PTP, causes release of cytochrome *c* (purple stars) and activation of caspase 9, and eventually commits cells to apoptosis [87]. Virus infection may generate either extrinsic or intrinsic apoptotic stimulus. For example, HIV-1 Tat-induced overexpression of tumor necrosis factor (TNF) and HCV core and NS5A-induced elevation of reactive oxygen species (ROS) may exacerbate mitochondrial Ca^{2+} overloading and cause apoptosis in infected cells. Intracellularly, abnormal increase of mitochondria Ca^{2+} uptake (e.g., HCV core protein [29,30]; red oval) and disruption of IMM permeability (e.g., HIV-1 Vpr [90], HTLV-1 p13[II] [92] and HBV HBx [31]; red ovals or cylinder) may also lead to apoptotic cell death. Viral proteins induce apoptosis to facilitate virion release and maximize virus dissemination at a later stage of viral life cycle. In contrast, actions that downregulate ER-mitochondria Ca^{2+} flux (e.g., pore-formation on ER by Coxsackievirus 2B [21,24]) or recruit pro-apoptotic protein (e.g., Bax) within mitochondria (e.g., CMV vMIA [26,27]), may protect host cells from apoptosis and promote cellular survival signaling [23]. In general, an anti-apoptotic strategy is employed by virus to aid virus replication and effective immune evasion in early or middle stages of infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Then comes a more important question: what purposes do alteration in $[Ca^{2+}]_{CYT}$ serve in the virus life cycle? First, a modestly elevated $[Ca^{2+}]_{CYT}$ would activate or accelerate a number of Ca^{2+}-dependent enzymatic processes in the cytosol, as well as Ca^{2+}-sensitive transcriptional factors (e.g., NFAT), to promote virus replication or to establish persistent infection (see Sections 2.1 and 2.2) (e.g., HIV-1 [12], HTLV-1 [13,14], HCV [15,16], HHV-8 [17]). Active Ca^{2+} (released from ER) uptake by mitochondria would boost ATP production to meet a higher demand for energy due to continuous viral replication (e.g., HCV [18], CMV [19]). Second, a decrease in $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_{Golgi}$ may inhibit protein trafficking pathways,

and thus thwart host anti-viral immune responses and escape premature clearance by the host. Moreover, intracellular accumulation of ER or Golgi-derived secretory vesicles, where viral RNA replication takes place for some RNA viruses, creates a microcosmic environment favoring viral replication (e.g., enterovirus [20,21]) (see Section 2.3.1). Third, modulation of ER-mitochondria Ca^{2+} coupling may either prevent apoptosis or induce apoptosis [22,23], all depending on the stages of the viral life cycle and types of viruses (see Sections 2.3 and 2.4). Apoptosis is usually elicited as an innate defense mechanism to counteract virus infection and control virus reproduction. On the one hand, viruses may target important apop-

totic checkpoints to prevent host immune clearance and promote virus replication (e.g., Coxsackievirus [21,24,25], CMV [26,27]). On the other hand, viruses may take approaches to accelerate or induce apoptotic cell death to facilitate virion release and maximize virus dissemination (e.g., HIV-1 [28], HCV [29,30], HBV [31]) (Fig. 2).

A detailed summary of virus-induced cellular Ca^{2+}-signaling alterations are listed in Supplementary Table 1. Instead of exhaustively describing how every virus perturbs the Ca^{2+} homeostasis (reviewed by Chami et al. [32]), this review will highlight the mechanistic similarity and common themes shared by different viruses to perturb the cellular Ca^{2+}-signaling network for the sake of their own benefit (Fig. 1).

2.1. Aberrant activation of plasma membrane Ca^{2+} entry channels

The plasma membrane serves as the very first barrier against virus infection. Various Ca^{2+} channels (voltage-operated [VOC], receptor-operated [ROC], or store-operated [SOC]) are responsible for feeding extracellular Ca^{2+} to the cytosol, whereas PMCA and the Na^+/Ca^{2+} exchanger extrude Ca^{2+} to the outside. During virus infection, these plasma membrane Ca^{2+}-signaling components often become the immediate target of attack.

2.1.1. VOC and ROC

VOC (e.g., L-type or $Ca_V1.4$) and ROC (e.g., N-methyl-D-aspartate [NMDA] receptor) are two of the most well studied targets due to the availability of specific channel blockers (e.g., verapamil and nifedipine). These channels are found in most excitable cells and a few non-excitable cells. Examples of virus-induced cytoplasmic Ca^{2+} immobilization via these channels can be found in retroviruses (e.g., HIV-1), RNA viruses (e.g., rotavirus) and DNA viruses (e.g., human Herpesviruses [HHV]).

Human immunodeficiency virus-1 (HIV-1) belongs to the *Retroviridae* family that has an RNA genome, but replicates through a DNA intermediate that integrates into the infected cell's DNA. Two HIV-1 proteins, the membrane glycoprotein gp120 and the transcriptional transactivator Tat, have been shown to induce the elevation of $[Ca^{2+}]_{CYT}$ in both excitable cells (such as neuronal cells) and non-excitable cells (such as immune cells and epithelial cells) by modulating the activity of VOC and/or ROC. External application of recombinant gp120 to fetal neurons and astrocytes causes a dose-dependent increase in $[Ca^{2+}]_{CYT}$ up to ∼2 μM and further induces neurotoxicity in these cells [33,34]. As demonstrated by using selective channel antagonists or blockers, the increase in $[Ca^{2+}]_{CYT}$ is attributable to the activation of L-type voltage-sensitive Ca^{2+} channels and NMDA receptors on the plasma membrane. In addition, similar gp120-induced elevation of $[Ca^{2+}]_{CYT}$ has been found in presynaptic terminals of rat cortical synaptosomes [35] and human intestinal epithelial cells HT-29-D4 [36], and the latter effect is related to the pathogenesis of HIV-1 related enteropathy. These findings suggest that drugs blocking gp120-induced intracellular Ca^{2+} changes in the human brain might be potential candidates for the treatment of HIV-1 dementia and enteropathy.

The HIV-1 protein Tat upregulates the viral gene expression and replication allowing HIV-1 to replicate in unactivated T cells. An increase in cytosolic Ca^{2+}, due to increased Ca^{2+} influx via NMDA type glutamate receptor and the release of Ca^{2+} from IP_3-sensitive internal stores via IP_3R, is observed in Tat-treated fetal neurons, astrocytes and microglia cells [37–39]. The Tat-induced dysregulation of intracellular Ca^{2+} leads to neurotoxicity and contributes to HIV-related dementia. In non-excitable human monocytes, Tat promotes extracellular Ca^{2+} influx to the cytoplasm via the dihydropyridine (DHP) sensitive L-type Ca^{2+} channel and further stimulates the production of pro-inflammation cytokine TNF-α [40]. TNF-α plays a major role in progression to AIDS by directly activating NF-κB to promote HIV-1 replication [41].

The tight link between Tat-induced Ca^{2+} mobilization and TNF-α production provides excellent opportunities to develop effective therapeutic approaches targeting Tat and DHP sensitive L-type Ca^{2+} channels to attenuate HIV-1 infection. Interestingly, studies in other immune cells suggest that HIV-1 Tat inhibits Ca^{2+} entry in human natural killer (NK) cells and dendritic cells (DC). Exogenous Tat impairs the cytotoxic activity of NK cells [42] and inhibits apoptotic body engulfment and IL-12 production in DC [43] by blocking L-type Ca^{2+} channel channels. Tat-induced opposition effects on L-type Ca^{2+} channels in different types of immune cells is not surprising, since both the stimulating effects on monocytes and the inhibitory effects on NK and DC synergically facilitate the efficient replication and immune evasion of HIV-1.

In addition to HIV-1, viruses harboring RNA genomes are also able to elicit alteration in $[Ca^{2+}]_{CYT}$ via plasma membrane Ca^{2+} entry pathways. Rotaviruses are members of the *Reoviridae* family that contain double-stranded RNA genomes and are the major etiological agent of viral gastroenteritis in young children. Ca^{2+} plays a key role in the replication of rotaviruses and pathogenesis of rotavirus infection [44]. Rotavirus-infected monkey kidney cells MA104 and the colon carcinoma HT29 cells have been shown to exhibit a progressive elevation of cytosolic Ca^{2+} due to increased membrane permeability at early times postinfection (4–8 hpi) [45]. The entry of Ca^{2+} is partially blocked by the L-type voltage-sensitive Ca^{2+} channels blocker metoxyverapamil (D600) in a dose-dependent manner. More importantly, the permeability pathway is selectively permeable for the passage of divalent ions that include Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+} and Co^{2+}, but is impermeable to the trivalent cations La^{3+} and Cr^{3+}. Such metal ion selectivity is observed commonly in divalent cation channels in both excitable and non-excitable cells [46]. These lines of evidence suggest that rotavirus-induced external Ca^{2+} entry pathway is characteristic of a Ca^{2+} channel rather than nonselective impairment of the plasma membrane. Such early activation of Ca^{2+} entry is speculated to maintain an elevated ER Ca^{2+} pool that is required for virion maturation and stability [47,48].

A similar scenario is also visualized in mammalian cells infected by DNA viruses. Epstein-Barr virus (EBV, or HHV-4), a member of the *Herpesviridae* family, large DNA viruses with an enveloped virion, is the etiological agent of infectious mononucleosis and a number of malignancies, such as Burkitt's lymphoma and nasopharyngeal carcinoma. In B lymphocytes, EBV infection can induce a rise in $[Ca^{2+}]_{CYT}$ due to increased Ca^{2+} influx from the extracellular space [49]. The rise in intracellular Ca^{2+} can be blocked by verapamil, a L-type Ca^{2+}-channel blocker [50]. In human fibroblasts infected by another herpesvirus, human cytomegalovirus (CMV, or HHV-5), a similar rise in $[Ca^{2+}]_{CYT}$ is also observed. This effect can be blocked by L-type Ca^{2+}-channel blockers nifedipine and verapamil [51]. A herpesvirus-induced increase in $[Ca^{2+}]_{CYT}$ and its association with B cell activation is speculated to favor transformation of B cells by efficiently integrating viral genes into the host cell genome.

2.1.2. SOC

In excitable cells, any external stimulus that depolarizes the plasma membrane is capable of activating VOC/ROC and leading to swift Ca^{2+} flux into cytoplasm. In contrast, only a few non-excitable cells possess VOC or ROC. Alternative Ca^{2+} entry pathways have to evolve to meet demands on Ca^{2+} in these cells. It has been noted for over two decades that the depletion of ER Ca^{2+} stores often triggers sustained Ca^{2+} entry across the plasma membrane. This Ca^{2+} permeabilization pathway, known as store-operated Ca^{2+} entry (SOCE) and characterized by a calcium release-activated calcium current (I_{CRAC}), tightly links intracellular Ca^{2+} release with Ca^{2+} influx and provides a means to refill the Ca^{2+} stores [52] (Fig. 2). However, the molecular basis responsible for SOCE has remained enigmatic during the past two decades. Not surprisingly, only a few studies reported virus-induced activation of SOCE. Among those, p12[I]

encoded by the retrovirus, human T-lymphotropic virus 1 (HTLV-1) [13] and the core protein encoded by the single-strand RNA flavivirus hepatitis C virus (HCV) [15] have been reported to induce ER Ca^{2+} depletion and further trigger opening of CRAC channels in T cells or Jurkat T cells. A sustained elevation of $[Ca^{2+}]_{CYT}$ and accompanying high frequency cytosolic Ca^{2+} oscillation specifically activate the nuclear factor of activated T-cells (NFAT), a transcriptional factor that initiates a highly coordinated choreography of gene expression [53]. Activation of NFAT exerts long-term effects on immune cells by inducing gene expression (e.g., IL-2 and Bcl-2) and promoting lymphocyte activation and survival, thereby supporting viral infection (Fig. 2). In addition, p12^I has also been shown to interact with Ca^{2+}-responsive transcription coactivator, p300, to mediate various transcription factors and further promote infected lymphocyte survival [54].

Recently, competitive and arduous searches for molecular identities of SOCE resulted in the discovery of ER Ca^{2+} sensor protein, stromal interaction molecule 1 (STIM1) [55,56], and the CRAC channel pore-forming subunit Orai1 [57–59] (Fig. 2). With these new players on hand, further investigations on how virus infection modulates SOCE await to be carried out at the molecular level in the immediate future.

2.1.3. PMCA

An alternative mechanism to sabotage Ca^{2+} homeostasis is achieved by targeting Ca^{2+} extrusion components, such as PMCA. Hepatitis B virus (HBV) is a simple virus (4 genes) belonging to the *Hepadnaviridae* family. Although HBV harbors a DNA genome, it replicates through an RNA intermediate and thus is more closely related to the retroviruses than to other DNA virus families. Overexpression of HBV X protein (HBx) in HepG2 and HeLa cells has been reported to stimulate caspase-3-dependent cleavage of PMCA, a pump that extrudes Ca^{2+} from cytoplasm to the outside [31]. The decrease in PMCA activity, as well as HBx-mediated release of mitochondrial Ca^{2+} [60], leads to a net increase in $[Ca^{2+}]_{CYT}$ that enhances HBV DNA replication [61] and favors HBx-mediated Ca^{2+}-responsive signaling pathways, including NFAT [62,63], SAPK/JNK and MAPK pathways [60]. These pathways are closely involved in the regulation of cell survival, apoptosis and proliferation. In addition, the rise in cytosolic Ca^{2+} triggers the activation of a Ca^{2+}-dependent proline-rich tyrosine kinase-2, which in turn activates the Src kinase and up-regulates reverse transcription [61]. More strikingly, such effects exerted by HBx can be substituted with simple increase in cytosolic Ca^{2+}, suggesting that Ca^{2+} is a fundamental requirement for HBV replication and infection. More than that, the HBX-induced increase in $[Ca^{2+}]$ has also been shown to enhance HBV core assembly [64]. Treatment of infected HepG2 cells with the cell-permeable Ca^{2+}-chelator BAPTA-AM and cyclosporine A results in reduced HBV capsid assembly, whereas the application of thapsigargin, a strong inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), increased HBV core assembly [64].

2.2. Activation of ER membrane Ca^{2+} release channels by viral proteins

The internal stores, such ER and Golgi complex, serve as another source of Ca^{2+} flux in mammalian cells. IP$_3$R and RyR are two of the central players in switching on/off the Ca^{2+} release from these internal stores [4]. The engagement of receptors on lymphocytes or agonist binding to cell surface receptors activates phospholipase C-γ (PLC-γ), which hydrolyzes phosphatidylinositol-4,5-biphosphate (PIP$_2$) to produce inositol-1,4,5-triphosphate (IP$_3$). IP$_3$ activates IP$_3$R, triggers Ca^{2+} release from stores and further increases IP$_3$R's sensitivity to Ca^{2+} (a bell-shape biomodal response) [65]. Low amounts of Ca^{2+} (<300 nM) released from ER further stimulate the activity of IP$_3$R. This calcium-induced Ca^{2+} release (CICR) mech-

anism enables Ca^{2+} signals to be rapidly amplified and spread throughout the whole cell. The replenishing of Ca^{2+} stores is achieved by Ca^{2+} influx across plasma membrane through SOCE and pumping of cytosolic Ca^{2+} back to lumen via SERCA pump. By virtue of ER's roles as a hub in coordinating the ebb and flow of intracellular Ca^{2+}, viruses containing either RNA or DNA genomes have evolved astute means to manipulate IP$_3$R-mediated Ca^{2+} mobilization (e.g., HIV-1, rotavirus, HSV) and/or affect the activity of SERCA pumps (e.g., HCV). Several key viral proteins have been reported to directly induce Ca^{2+} release from Ca^{2+} stores.

2.2.1. HIV-1 Nef

HIV-1 Nef is the major player downregulating the immunologically important cell surface proteins, CD4 and MHC-I [66]. Nef may induce a cytosolic Ca^{2+} increase in Jurkat T cells through its interaction with IP$_3$R and subsequently promote the T cell receptor-independent activation of the NFAT pathway, without notable increase in PLCγ1-catalyzed production of IP$_3$ [67]. The activated NFAT, a transcriptional factor responding to the low-amplitude intracellular Ca^{2+} oscillation, can further promote the viral gene transcription and replication [12]. The increase in cytosolic Ca^{2+} by Nef has also been demonstrated in differentiated myelomonocytic HL60 cells [68]. In addition, it is interesting to note that a Src-like protein-tyrosine kinase (PTK) coimmunoprecipitate with both Nef and the IP$_3$R. Given this, it is hypothesized that Nef might modulate the activity of the IP$_3$R activity via its interaction with the Src-like PTK [68].

2.2.2. HCV core protein

HCV is a member of the *Flaviviridae*, a family of positive polarity, single-strand RNA viruses. Transient and stable expression of the HCV core protein in Huh7 cells, a human liver carcinoma line universally used to study HCV replication, induces ER Ca^{2+} depletion by impairing the function of SERCA pump [29]. The inhibition of SERCA activity is possibly caused by overexpression of inducible nitric oxide synthase and calreticulin in response to HCV core-induced ER stress. HCV core-induced ER stress further activates the pro-apoptotic Bcl2-associated X protein (Bax) [29,30] (Fig. 2), which induces opening of the mitochondrial voltage-dependent anion channel, cytochrome *c* release, apoptosis, and eventually, liver damage and even oncogenesis [69].

2.2.3. Rotavirus nonstructural protein 4 (NSP4)

The rotavirus nonstructural glycoprotein NSP4 acts as a multifunctional enterotoxin [70] and has been shown to induce Ca^{2+} mobilization in infected human epithelial cells through a PLC-dependent pathway [71]. Exogenously applied rotavirus nonstructural protein NSP4 can induce diarrhea in rodent pups and increases cytosolic Ca^{2+} concentration via the activation of PLC and the resultant ER Ca^{2+} depletion through IP$_3$R [71]. In addition, endogenous NSP4 can also be secreted from the apical surface of polarized epithelial cells [72] or released outside after cell lysis, thus exerting exogenous action on neighboring non-infected cells. Several lines of evidence suggest that the NSP4-induced Ca^{2+} mobilization is linked to the stimulation of chloride secretion and diarrhea [73]. However, the underlying molecular mechanisms are still not clear. In this regard, more investigation is needed to elucidate the mechanisms underlying the NSP4-induced alteration of intracellular Ca^{2+} homeostasis and its correlation with chloride secretion in rotavirus-infected cells.

2.2.4. HSV particles

Exposure of epithelial cells (CaSki and Vero) to herpes simplex viruses 1 (HSV-1/HHV-1) and HSV-2 (or HHV-2) has been shown to result in a rapid and transient increase in intracellular Ca^{2+} by triggering ER Ca^{2+} release via IP$_3$R but not a Ca^{2+} influx across the

plasma membrane [74]. The viral protein responsible for immobilizing intracellular Ca^{2+} remains to be elucidated. The increase in $[Ca^{2+}]_{CYT}$ in turn triggers phosphorylation of focal adhesion kinase (FAK) 5–10 min after virus exposure [74]. Notably, FAK phosphorylation has been demonstrated to promote actin cytoskeleton reorganization and trafficking of incoming virus particles. In this way, altered Ca^{2+} signaling is directly linked to a phosphorylation event and plays a central role in facilitating early events in HSV-1/HSV-2 invasion.

2.3. Direct permeabilization of ER/Golgi by pore-forming viral proteins

As the major organelle involved in regulating Ca^{2+} homeostasis and signaling, the Ca^{2+} filling status within ER is closely associated with apoptosis [22,23]. Ca^{2+} released from ER can be readily taken up by mitochondria in close proximity to the extruding mouth. Abnormal exodus of ER Ca^{2+} could result in Ca^{2+} overloading in mitochondria and induce the opening of the mitochondrial permeability transition pore and subsequent release of cytochrome c from the mitochondria, which activates caspase 9 and commits cells to apoptosis (Fig. 2). In contrast, a down-regulated ER-mitochondria Ca^{2+} signaling would reduce the Ca^{2+} level in mitochondria and promote the cell survival pathway [23]. Thus, Ca^{2+} flux between ER and mitochondria plays a critical role in determining the fate of host cells when exposed to apoptotic stimuli, such as virus infection. Aside from altering the ER Ca^{2+} store level via ER Ca^{2+} release channels as described above, viruses exploit alternative ways to adjust Ca^{2+} store filling status by directly forming pores on membrane or disrupting membrane permeability of Ca^{2+} stores. This is best exemplified by five viral proteins encoded by RNA (e.g., enterovirus, HCV, and rotavirus) or DNA viruses (e.g., CMV).

2.3.1. 2B protein (enteroviruses)

Polioviruses and Coxsakievirus are typical of the non-enveloped enterovirus genus in the *Picornaviridae* family. The single-stranded, plus-sense RNA genome encodes 4 structural proteins (VP1-4) and 10 nonstructural proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$, $3D^{pol}$, 2BC, 3AB, and $3CD^{pro}$) that are produced by a proteolytic processing cascade from a single translational precursor. Among these, the 2BC polypeptide of poliovirus has been shown to induce an increase of $[Ca^{2+}]_{CYT}$ at 2–4 hpi [75,76]. This time point is coincident with a period during which the viral proteins are actively synthesized. Also, the 2B protein of Coxsakievirus has been shown to directly cause decrease of Ca^{2+} concentrations in subcellular compartments, such as Golgi complex and ER, by forming pores on the membranes of these organelles and subsequently causing Ca^{2+} efflux from the lumen of these organelles. The 2B protein is characteristic of viroporin, a group of integral membrane proteins containing amphipathic α-helices and capable of forming pores on membrane to aid virion production and dissemination [77]. 2B has been demonstrated to multimerize and form hydrophilic pores when incorporated into liposomes with an estimated pore size of 6 Å. The aqueous pores on the membrane allow solutes with molecular mass <1 kDa to pass through freely [78]. The metal selectivity and electrophysiological properties of reconstituted 2B pores is still under close scrutiny.

The 2B-induced, substantial decrease (∼40%) of Ca^{2+} in ER and Golgi complex serves two purposes: (i) it inhibits intracellular protein trafficking pathways, thus favoring virus replication and down-regulating host anti-viral immune response [79]. A reduced intravesicular Ca^{2+} level would result in the inhibition of vesicular protein transport and accumulation of ER/Golgi-derived vesicles, where the replication complex forms and viral RNA replication takes place. (ii) It exerts an anti-apoptotic activity on infected cells. Upon enterovirus entry, the cellular apoptotic process is imme-

diately triggered as an innate defense mechanism in response to infection, but is abruptly suppressed during the middle stage of infection. Overexpression of 2B in HeLa cells renders these cells resistant to cycloheximide and actinomycin D-induced apoptosis. The apoptotic process resumes at late stage after viral replication. The perturbation of Ca^{2+} homeostasis at 2–4 hpi coincides with the inhibition of the apoptotic cell response triggered at about 2 hpi. Thus, the middle-stage interruption of apoptosis is linked to down-regulated ER-mitochondrial Ca^{2+} fluxes that prevent cytotoxic Ca^{2+} overloading in mitochondria. In summary, 2B-induced reduction of Ca^{2+} in ER/Golgi provides the virus a favorable cellular environment and optimal time-window for viral RNA and protein synthesis. Thus, the exquisite synchronization of $[Ca^{2+}]_{CYT}$ increase and anti-apoptotic activity allows virus particles to complete assembly before cell lysis [21,24,25].

2.3.2. p7 and NS5A (HCV)

The HCV viroporin p7, a 63-residue hydrophobic and poorly characterized protein found in the ER membrane, forms hexamers on artificial lipid membranes and functions like a Ca^{2+} ion channel [80]. These findings suggest that p7 might be responsible for the flow of Ca^{2+} from ER to the cytoplasm in HCV-infected cells. The reconstituted channel can be blocked by the anti-viral drug amantadine or long-alkyl-chain iminosugar derivatives [80], which raises the possibility of aiming at p7 as a potential therapeutic target. Another HCV nonstructural protein NS5A, which is embedded on the ER membrane, has been shown to alter the intracellular Ca^{2+} levels by increasing ER Ca^{2+} efflux [81]. The released Ca^{2+} can be readily taken up by mitochondria and increase the production of mitochondrial reactive oxygen species (ROS). Elevated ROS leads to a shift in cellular redox state and activates transcription factors including NF-κB and STAT3, which promote cell growth and proliferation and protect hepatocytes from apoptosis, thereby preventing premature abortion of the virus life cycle [81].

2.3.3. NSP4 (rotavirus)

Aside from the exogenous NSP4-induced, PLC-dependent Ca^{2+} immobilization pathway (described above) [71], PLC-independent pathways also play important roles in inducing elevation of intracellular Ca^{2+} [82]. The glycoprotein NSP4 is found to be primarily embedded in the ER membrane of rotavirus-infected cells [83]. Endogenously expressed NSP4 in Sf9 insect cells and HEK293 can alter the ER membrane permeability and therefore cause a sustained increase of cytosolic Ca^{2+} concentration that is independent of the PLC pathway [71,82]. By using a liposome leakage assay that monitors the release of fluorescent dye from calnexin-loaded liposomes mimicking ER membrane, the membrane destabilization activity of NSP4 is mapped to residues 114–135 [84]. This membrane destabilization activity (MDA) seems to be quite specific to ER, since neither the full NSP4 protein nor the NSP4 fragment (114–135) exerts MDA on plasma membrane.

2.3.4. vMIA or pUL37 × 1 (CMV)

The CMV-encoded protein pUL37 × 1 (also called the mitochondria-localized inhibitor of apoptosis, or vMIA) is capable of trafficking from the ER to mitochondria in CMV-infected HeLa cells and human fibroblasts. Expression of pUL37 × 1 has been shown to induce Ca^{2+} release 4-6 hpi from the ER store with concomitant cell morphological changes, such as cell rounding, cell swelling, actin cytoskeleton reorganization and mitochondrial fission [19]. The altered Ca^{2+}-signaling has two cytobiological consequences: (i) the Ca^{2+} released from ER is taken up by nearby mitochondria and accelerates energy metabolism to generate sufficient ATP for active virus replication by activating Ca^{2+}-dependent mitochondrial enzymes. (ii) A lower ER Ca^{2+} store level exerts protective effects on ceramide-induced apoptosis [22,23]. In addition,

vMIA directly interacts with the host pro-apoptotic protein Bax via its C-terminal domain (residues 115–147) and recruits Bax from cytosol to mitochondria [26]. In this way, vMIA sequesters Bax at the mitochondrial membrane in an inactive form and blocks Bax-mediated apoptosis [26,27].

2.4. Disruption of mitochondrial membrane permeabilization and/or potential

The multifunctional organelle mitochondrion is an integral part of the internal Ca^{2+} pool and functions as a hub of energy production and apoptotic cell death. In mitochondria, Ca^{2+} can easily pass through outer mitochondrial membrane (OMM) pores and cross the inner mitochondrial membrane (IMM) through membrane-embedded channels and transporters. The resting inner membrane potential of the mitochondrion ($\Delta\psi_m$) is maintained at -150 to -180 mV by actively pumping protons across the inner membrane. Disruption of membrane permeability and dissipation of $\Delta\psi_m$ may lead to ATP depletion and cell death. Mitochondrial Ca^{2+} uptake is mediated by the mitochondrial voltage-dependent anion channel (VDAC) across OMM and the $\Delta\psi_m$-driven Ca^{2+} uniporter of IMM. The Ca^{2+} uniporter is a highly selective, saturable channel with low affinity for Ca^{2+} ($\sim 10\,\mu M$). Ca^{2+} exits mitochondria through the opening of a non-selective high-conductance channel, permeability transition pore (PTP) in IMM and the Na^+/Ca^{2+} exchanger [85,86] (Fig. 2). The IMM protein adenine nucleotide translocator (ANT) also contributes to the permeability transition. An increase in matrix Ca^{2+} may activate Ca^{2+}-dependent Kreb's cycle dehydrogenases and increase production of ATP to restore $\Delta\psi_m$. However, excess Ca^{2+} in mitochondrial matrix is pro-apoptotic since it activates the opening of PTP and results in loss of $\Delta\psi_m$ and release of cytochrome *c* [87]. In contrast, actions that reduce matrix Ca^{2+} (e.g., downregulation of ER/mitochondria Ca^{2+} flux) may protect host cells from apoptosis [23]. During virus infection, viral proteins may readily target mitochondria and exert either pro-apoptotic or anti-apoptotic action by altering mitochondrial Ca^{2+} signaling in host cells, depending on the stages of the viral life cycle. In general, an anti-apoptotic strategy is employed by the virus to prevent host immune clearance and promote virus replication in the early or middle stage of infection. Meanwhile, virus infection may induce apoptosis to aid egress of virions to the outside and dissemination of progeny at a later stage.

2.4.1. Vpr (HIV-1)

HIV-1 viral protein R (Vpr) is a 14-kDa multifunctional protein localized in nucleus and mitochondria of HIV-1 infected cells. Vpr is known as a pro-apoptotic reagent. In either isolated mitochondria or cells, Vpr tightly interacts with ANT and induces mitochondrial membrane depolarization, Ca^{2+} leakage and cytochrome c release from mitochondria [88,89]. Indeed, a C-terminal fragment of Vpr (resides 52–96), along with ANT, has been shown to form ion channels in synthetic membrane and control mitochondrial membrane permeabilization [89]. In addition, Vpr has been detected in nucleus and may activate the Ca^{2+}-responsive transcriptional coactivator p300 (similar to p12[I] of HTLV-1 [54]) and CREB-binding protein, thereby inducing HIV-1 transcription [90,91].

2.4.2. p13[II] (HTLV-1)

The HTLV-1 accessory protein p13[II] is an 87-amino-acid mitochondrial protein primarily located at IMM [92]. P13[II] contains a potent mitochondria targeting sequence (residues 21–31) that forms an amphipathic α-helix [93]. p13[II] causes swelling and depolarization of mitochondria by increasing inner membrane permeability to cations, such as Ca^{2+}, Na^+ and K^+ [94]. Such changes are believed to be responsible for promoting ceramide- or Fas ligand-induced apoptosis in T lymphocytes expressing p13[II] [92].

The change in membrane permeability is independent of PTP with neither release of cytochrome *c* nor commitment to apoptosis [93]. Moreover, Ca^{2+} uptake is not inhibited by the addition of ruthenium red (a Ca^{2+} uniporter antagonist), suggesting that Ca^{2+} uniporter does not play a role in Ca^{2+} flux [94]. All these findings indicate that p13[II], like HCV p7 [80] and Coxsakievirus 2B [77], might function as a viroporin and alter membrane permeability by directly forming pores on IMM. This hypothesis is under intensive scrutiny and needs to be validated at the molecular level.

2.4.3. Core protein (HCV)

The HCV core protein has been shown to increase mitochondrial ROS production and enhance Ca^{2+} uptake in mitochondria [18]. The increase in steady-state mitochondrial Ca^{2+} concentration is ascribed to an enhanced activity of Ca^{2+} uniporter but not because of an impaired function of the Na^+/Ca^{2+} exchanger [18]. By increasing mitochondrial Ca^{2+} uptake, HCV may stimulate ATP production, increase ROS production, and induce apoptosis of HCV-infected cells. These detrimental effects are linked to the pathogenesis of the chronic liver disease and carcinogenesis associated with HCV-infection [18].

2.4.4. HBx (HBV)

In addition to the aforementioned capability to impair the Ca^{2+} extruding pump PMCA, the multifunctional HBx colocalizes with VDAC3 and is linked to apoptosis by interrupting ER/mitochondria coupling and inducing cytochrome c release from mitochondria [31]. All these mechanisms lead to an elevated level of $[Ca^{2+}]_{CYT}$, which is found to benefit viral replication and core assembly [61,64].

Overall, different viral proteins may perturb the Ca^{2+}-signaling networks by targeting the same Ca^{2+}-signaling components at different subcellular compartments. Moreover, a single viral protein (e.g., Tat, NSP4, core protein and HBx) can manipulate multiple Ca^{2+} signaling mechanisms by targeting different Ca^{2+} signaling components. In this way, a virus makes full use of its limited gene products to hijack the cellular Ca^{2+} signaling machinery for its own benefit.

3. Viral Ca^{2+}-binding proteins

To date, the majority of the reported viral Ca^{2+}-binding proteins (CaBPs) are structural proteins, including both coat and envelope proteins. In the former category, Ca^{2+} is required to maintain the structural integrity and/or the proper assembly and disassembly of virions (examples include turnip crinkle virus [95,96], tobacco mosaic virus [97,98] rotavirus [99–102], polyomaviruses [98,103], and HBV [64]). Examples of the latter category include the HIV-1 envelope glycoprotein, gp 160, which requires Ca^{2+} binding for cell fusion [104,105] and influenza B virus neuraminidase, Ca^{2+} binding to which enhances both stability and enzymatic activity [106,107]. Ca^{2+}-binding sites in viral CaBP's can be divided into two types: discontinuous and continuous. While both types of Ca^{2+}-binding sites have been found in the proteins of diverse virus families, more is known about virus proteins with discontinuous binding sites (Supplementary Table 2). However, as will be presented, a bioinformatics approach reveals that putative continuous Ca^{2+}-binding sites are also ubiquitous among virus proteins (Supplementary Table 3).

3.1. Discontinuous viral Ca^{2+}-binding sites

In a discontinuous Ca^{2+}-binding site, the Ca^{2+}-binding pocket is formed by distant ligands within the primary sequence or on different polypeptides. In the Protein Data Bank (PDB), 41 ($\sim 3.3\%$) hits were detected with the ligand name defined as "Ca^{2+}" in a total of 1200 viral protein structures (excluding redundant structures with >90% sequence similarity). However, the identification
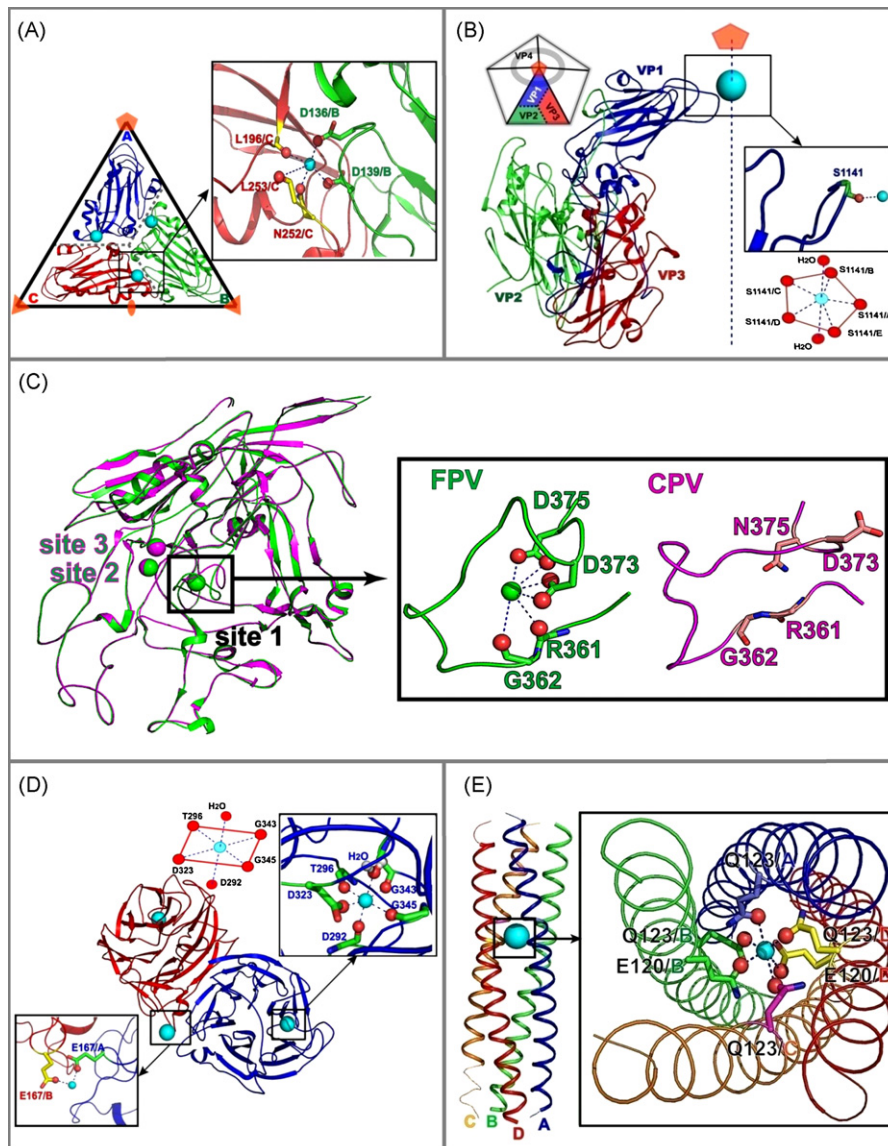
Fig. 3. Examples of viral Ca^{2+}-binding proteins. (A) 3D representation of the icosahedral asymmetric unit of the cocksfoot mottle virus capsid and the location of the incorporated Ca^{2+} ions (PDB code: 1ng0) [108]. The assembling unit is formed by three subunits, A (blue), B (green) and C (red) that are chemically identical but slightly different in conformational arrangement. Ca^{2+}, situated between the interfaces of neighboring subunits (A–B, A–C or B–C), is coordinated by oxygen atoms from the side chains of D136 and D139 in one subunit and oxygen atoms from the main chain of L196, the side chain of N252, and the C-terminal carboxyl group of L253 in the other neighboring subunit (enlarged area). The solid pentagon, triangle and oval represent 5-, 3-, and 2-fold axes of the icosahedron. (B) Ca^{2+} ion located on the 5-fold axis of the capsid of human rhinovirus 3 (HRV3) (PDB code: 1rhi) [112]. The icosahedral capsid of HRV3 is composed of 60 copies of each of the four capsid proteins VP1 (blue), VP2 (green), VP3 (red) and VP4 (black). VP1, VP2 and VP3 are exposed to the external surface of the viral particle, whereas VP4 lies in the internal surface. A Ca^{2+} ion is found situated on the 5-fold axis of the capsid and coordinated by 5 oxygen atoms from the main chain carbonyl group of the 5-fold symmetry-related S1141 on VP1 (enlarged area). With two additional oxygen atoms from water molecules above or below the metal ion as coordinating ligands, the Ca^{2+}-binding pocket forms a pentagonal bipyramidal geometry. (C) Overlay of 3D structure of the virion protein VP2 of the single-stranded DNA-containing parvoviruses, feline panleukopenia virus (FPV; PDB code: 1C8F; green) and canine parvorius (CPV; PDB code: 1C8D; magenta). The VP2 proteins of FPV and CPV bind three and two Ca^{2+} ions, respectively, at pH 7.5 [117] (Fig. 2C). Both FPV and CPV contain pH-dependent dual Ca^{2+}-binding sites (sites 2 and 3; spheres with mixed green and magenta colors), in which the metal ions are separated by 4.6 Å. The most striking structural difference between VP2 of these two viruses is within a flexible surface loop located within residues 359–375. This loop forms a third Ca^{2+}-binding site in FPV (site 1; green sphere), but not in CPV since D375 is replaced by N375 in CPV. This unique Ca^{2+}-binding site in FPV VP2 is coordinated by oxygen atoms from the side chain of residues D373 and D375, as well as the main chain carbonyl groups of residues R361 and G362. The binding of Ca^{2+} to this particular site is speculated to cause conformational changes in VP2 and may influence the host range of these two related, but different, parvoviruses. (D) The 3D structure of neuraminidase of influenza B virus (PDB code: 1nsb) [106]. The cartoon only represents half of the tetrameric form of this enzyme. Three Ca^{2+}-binding sites are found in two identical subunits, A (blue) and B (red). Each subunit contains one octahedral Ca^{2+}-binding site (upper panel) that involves residues D292, T296, D232, G343, G345 and one additional water molecule. Another site (lower panel), coordinated by the 4-fold symmetry-related E167, holds the oligomer together. (E), The core Ca^{2+}-binding pocket in the oligomerization domain (aa. 95–137) of NSP4 from rotavirus (PDB code: 2o1j) [123]. The domain self-assembles into a paralleled tetrameric coiled-coil. Chains A, B, C and D are shown in blue, green, orange and red, respectively. The Ca^{2+} ion is coordinated by six oxygen atoms from the side chains of Q123 on chains A to D, as well as the side chains of E120 on chains B and D. Calcium ions are shown as cyan spheres. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of discontinuous Ca^{2+}-binding sites relies solely on the determination of 3D structure; therefore, it is likely that Ca^{2+} ions might be a structural feature of more viral proteins, but this limitation impedes their discovery. Such Ca^{2+}-binding sites are most frequently found in the virus coat proteins, including those of both RNA viruses (e.g., enteroviruses, rhinoviruses, sobemoviruses and rotaviruses) and DNA viruses (e.g., parvoviruses and polyomaviruses). In addition, discontinuous sites are also found in the envelope-associated

neuraminidase protein of Influenza B viruses and the nonstructural NSP4 protein of rotaviruses (Fig. 3 and Supplementary Table 2).

3.1.1. Coat proteins

Among viruses whose coat proteins are CaBP's, viruses with both helical and icosahedral symmetry are represented. Tobacco mosaic virus, a typical helical virus, has been shown to bind Ca^{2+} with apparent affinities <100 μM [97,98]. With the extracellular Ca^{2+} concentration at the mM range, such an affinity would ensure a tight binding of Ca^{2+} to capsid proteins. Among viruses with icosahedral symmetry, the numbers of bound Ca^{2+} ions and coordinating geometry of the Ca^{2+}-binding sites differ in that the Ca^{2+} ions may be situated between the interacting interfaces of capsid subunits (Fig. 3A) or sit on the symmetric 3-fold or 5-fold axis (Fig. 3B). For example, the virion of cocksfoot mottle virus (CfMV), a plant *Sobemovirus* with a single-stranded, positive-sense RNA genome, has an icosacahedral capsid composed of 180 copies of the coat protein monomer assembled in $T = 3$ quasi-equivalent symmetry [108]. The monomer, which contains 253 amino acids, has a jelly-roll β-sandwich topology and can assume one of three slightly different conformations, denoted as quasi-equivalent A, B and C subunits. These subunits are assembled into asymmetric units which then coalesce to form the icosahedral capsid. Three Ca^{2+} ions, each incorporated between the interacting surfaces of subunits (A–B, B–C and A–C), function as reusable "glue" to stick adjacent subunits together and stabilize the capsid. Each Ca^{2+} ion is coordinated by Oδ1 of residues D136 and D139 from one subunit and the main chain carbonyl oxygen of L196, Oδ1 of N252, the C-terminal carboxyl oxygen of L253 from the other interacting subunit (Fig. 3A). Such Ca^{2+}-binding sites seem to adopt an octahedral geometry with only five protein ligands and an average Ca–O distance of 2.4 Å [108–110].

A second example of the incorporation of Ca^{2+} ions into an icosahedral virus particle are three members of the human rhinoviruses, all belonging to the *Picornavirus* family. In the virions of human rhinovirus 1A (HRV1A), HRV3 and HRV14, Ca^{2+} ions are located at the 5-fold axes of symmetry (Fig. 3B). In both HRV14 [111] and HRV3 [112], the metal ions are coordinated by the oxygen atoms from the main chain carbonyl group of S1141 in the five copies of virion protein (VP) 1 that meet at the 5-fold axis of symmetry. In HRV1A, the Ca^{2+} is coordinated by the Oδ1 of Asp1137 of the five copies of VP1 at the 5-fold axis [113]. With two additional oxygen atoms from water molecules above or below the metal ion as coordinating ligands, the Ca^{2+}-binding pocket forms a pentagonal bipyramidal geometry with Ca–O distance ranges from 2.3 to 3.7 Å. In HRV14, an additional Ca^{2+} ion is situated on the 3-fold axis of symmetry and is coordinated by the Oε1 of E3200 of VP3. Similar to HRV14, each asymmetric unit of the icosahedral capsid of Coxsachievirus B3, another *Picornavirus*, contains two Ca^{2+} ions, one interacting with Oδ1 of D3203 from VP3 on the 3-fold axis and the other making water-mediated interaction with the 5-fold axis-related residue D1133 on VP1 [114].

Aside from participating in virion structure and stability, examples of functionality of Ca^{2+} binding by virus coat proteins include the single-stranded DNA bacteriophage phiX174 which contains three repeating Ca^{2+}-binding sites within its icosahedral particle. Site 1, located ~10 Å off the 3-fold axis of symmetry, is composed of six ligands from the coat protein, F. Site 2 within the F protein and Site 3 within the G spike protein are on the 3-fold and 5-fold axes of symmetry, respectively. While the binding of Ca^{2+} ions to Site 2 and Site 3 stabilizes the capsid, binding to Site 1 is functionally important in attachment to the host cell and injection of viral DNA [115,116]. Similarly, the virion proteins of the animal single-stranded DNA-containing parvoviruses, feline panleukopenia virus (FPV) and canine parvorius (CPV), bind three and two Ca^{2+} ions, respectively [117] (Fig. 3C). CPV emerged in 1978 as a host range variant of FPV. Both FPV and CPV contain a pH-dependent dual

Ca^{2+}-binding site in which the metal ions are separated by 4.6 Å. The primary structural difference between the virion proteins of these two viruses is within a flexible surface loop located within residues 359–375 of the coat protein VP2 which is thought to interact with surface molecules on the host cell. This loop forms a third Ca^{2+}-binding site (Site 1, Fig. 3C) in FPV, but not in CPV. The binding of Ca^{2+} to this particular site causes conformational changes in VP2 and may influence the host range of these related, but different, parvoviruses [117].

The icosahedral virion of simian virus 40 (SV40), a member of the *Polyomavirus* family, a family of double-stranded DNA viruses, contains repeating dual Ca^{2+}-binding sites in its major coat protein, VP1. Mutations in the Ca^{2+}-binding ligands lead to defects in capsid assembly at nonpermissive temperature [118]. Nevertheless, some Ca^{2+} mutants were capable of forming virus-like particles (VLPs); however these were found to be less infectious than wild type VLPs. Interestingly, some of the mutants fail to enter the cell (e.g., E330K in Site 1) while others enter the cells, but fail to migrate to the nucleus (e.g., E157A-E160A and E216K in Site 2) due to an inability to bind Ca^{2+} leading to premature dissociation. Thus, the binding of two Ca^{2+} ions to the dual Ca^{2+}-binding site on VP1 is essential for the assembly of the icosahedral capsid, as well as the cell entry and nuclear transport [103,119].

3.1.2. Envelope proteins

An example of Ca^{2+} binding by a virion envelope protein is the neuraminidase of ortho- and paramyxo viruses, both families of negative-polarity, single-stranded RNA viruses of animals. The neuraminidase catalyses the cleavage of the glycosidic linkages between the terminal siliac acid residues and the body of carbohydrate moieties on the surface of infected cells. This activity is required for the release of virus from the cell surface. Two distinct Ca^{2+}-binding sites are found in the neuraminidase [106,120]. One high-affinity site (Fig. 3D) is close to the active site and adopts an octahedral geometry with six coordinating ligands including the carboxyl group of the side chain of D323, the carbonyl groups of the main chains of G343, G345, T296, D292 and one molecule of water. This Ca^{2+}-binding site is conserved among influenza A virus [120,121], influenza B virus [106] and the parainfluenza viruses [122]. The other site is a relatively low-affinity site, located on the 4-fold axis of the tetrameric neuraminidase of the influenza B viruses. The high-affinity Ca^{2+}-binding site is needed for the thermostability and optimal activity of the enzyme, whereas the low-affinity site has been postulated to hold the tetramer together [107].

3.1.3. Nonstructural proteins

The rotavirus NSP4, a transmembrane glycoprotein primarily embedded in the ER membrane of rotavirus-infected cells, is required for the budding of immature viral particle into the ER lumen and thus plays a central role in the morphogenesis of rotaviruses, even though it is not incorporated into the virus particle [44]. NSP4 also functions as an enterotoxin, triggering ER Ca^{2+} release, as discussed above, that eventually results in gastrointestinal symptoms [82,99]. NSP4 itself contains a core Ca^{2+}-binding site when it oligomerizes into a functional homo-tetramer [123]. The Ca^{2+} ion is coordinated by the side chains of four Q123 and two E120 residues from the four identical polypeptides within the tetramer (Fig. 3E). Ca^{2+}-binding at this site appears to stabilize the NSP4 tetramer [71].

3.2. Continuous viral Ca^{2+}-binding sites

The prototype continuous Ca^{2+}-binding site is the 29-residue EF-hand motif. The motif contains a helix-loop-helix topology, much like the spread thumb and forefinger of the human hand, in which
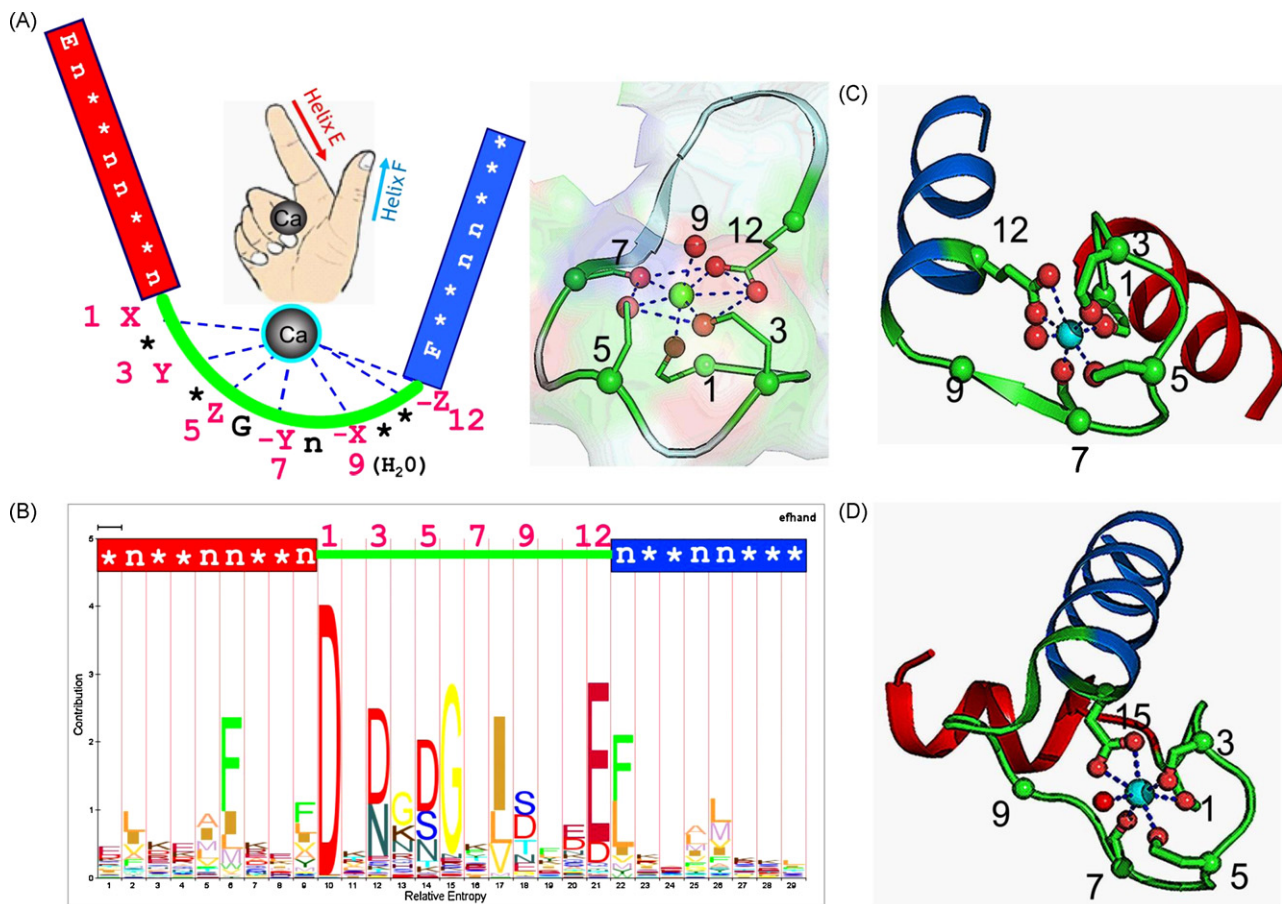
Fig. 4. The helix-loop-helix EF-hand Ca^{2+}-binding motif. (A) Cartoon illustration of the canonical EF-hand Ca^{2+}-binding motif. The EF-hand motif contains a 29-residue helix-loop-helix topology, much like the spread thumb and forefinger of the human hand. Ca^{2+} is coordinated by ligands within the 12-residue loop, including seven oxygen atoms from the sidechain carboxyl or hydroxyl groups (loop sequence positions 1, 3, 5, 12), a main chain carbonyl group (position 7), and a bridged water (via position 9). Residue at position 12 serves as a bidentate ligand. The Ca^{2+}-binding pocket adopts a pentagonal bipyramidal geometry. n stands for hydrophobic residue. (B) HMM logo for the canonical EF-hand motif (http://pfam.sanger.ac.uk/family?acc=PF00036). The conservation of amino acids at several positions makes it possible to predict EF-hand motifs from genomic sequences. (C) 3D structure of a typical canonical EF-hand motif from calmodulin (PDB code: 3cln). Ca^{2+} is chelated by ligands from a 12-residue loop. (D), 3D structure of an EF-hand-like motif from a soluble fragment of lytic transglycosylase B of *Escherichia coli* (PDB code: 1qut). This motif contains a 15-residue (instead of 12-residue) Ca^{2+}-binding loop flanked by two helices.

the Ca^{2+} ions are coordinated ligands within the loop, including seven oxygen atoms from the sidechain carboxyl or hydroxyl groups (loop sequence positions 1, 3, 5, 12), a main chain carbonyl group (position 7), and a bridged water (position 9) (Fig. 4A and B) [124]. The Ca^{2+}-binding pocket thus adopts a pentagonal bipyramidal geometry [125]. The EF-hand motif is one of the most widely used motifs among proteins and is distributed across bacteria, archaea, and eukaryotes [126,127]. Upon binding to Ca^{2+}, this motif may undergo conformational changes that enable Ca^{2+}-regulated functions as seen in Ca^{2+} effectors such as calmodulin (CaM) (Fig. 4C) and troponin C (TnC) and Ca^{2+} buffers such as calreticulin and calbindin D9k. While the majority of the known EF-hand CaBPs contain paired EF-hand motifs, CaBP's with single EF hands have also been discovered in both bacteria and eukaryotes [127,128]. In addition, "EF-hand-like motifs" have been found in a number of bacteria [127]. Although their coordination properties remain similar to the canonical 29-residue helix-loop-helix EF-hand motif, the EF-hand-like motifs differ from EF-hands in that they contain deviations in the secondary structure of the flanking sequences and/or variation in the length of the Ca^{2+}-coordinating loop (Fig. 4D).

Though EF-hand-containing proteins have been found abundantly in eukaryotes and bacteria, the literature on EF-hand or EF-hand like Ca^{2+}-binding motifs in virus proteins is thin, possibly due to lack of accurate prediction methods and robust methodologies for validation. A thorough search in PubMed with the key words

"EF-hand and virus" only resulted in four hits of predicted canonical EF-hand motifs: the transmembrane protein gp 41 of HIV-1 [104], VP1 of polyomavirus [129], the VP7 outer capsid protein of rotavirus [102], and the protease domain of rubella virus (discussed below) [130]. Recombinant VP1 of an avian polyomavirus that lacks the 12-residue predicted EF-hand loop is unable to bind ^{45}Ca and fails to assemble capsomeres into capsid-like particles [129]. However, the structural and Ca^{2+}-binding properties of the first three reported putative canonical EF-hands have not been characterized yet.

Our laboratory has developed a pattern search algorithm to predict continuous Ca^{2+}-binding sites from genomic information (http://www.chemistry.gsu.edu/faculty/Yang/Calciomics.htm). This tool has been successfully applied to predict and analyze potential EF-hand and EF-hand like Ca^{2+}-binding motifs in bacterial proteins at a genome-wide level [127]. Given the widespread occurrence of EF-hand motifs in cell proteins and the importance of Ca^{2+} as an intracellular messenger, it is surprising that only four of these motifs were reported in viral proteins. We therefore initiated a comprehensive search for potential viral EF-hand motifs by screening all of the viral genomic information available on the protein database Swiss-Prot/TrEMBL with both our algorithm [127] and the prediction motif signature PS00018 (http://ca.expasy.org/prosite/PDOC00018) from the Expert Protein Analysis System (ExPASy) proteomic server and detected an additional 93 putative EF-hand and EF-hand-like motifs (summarized
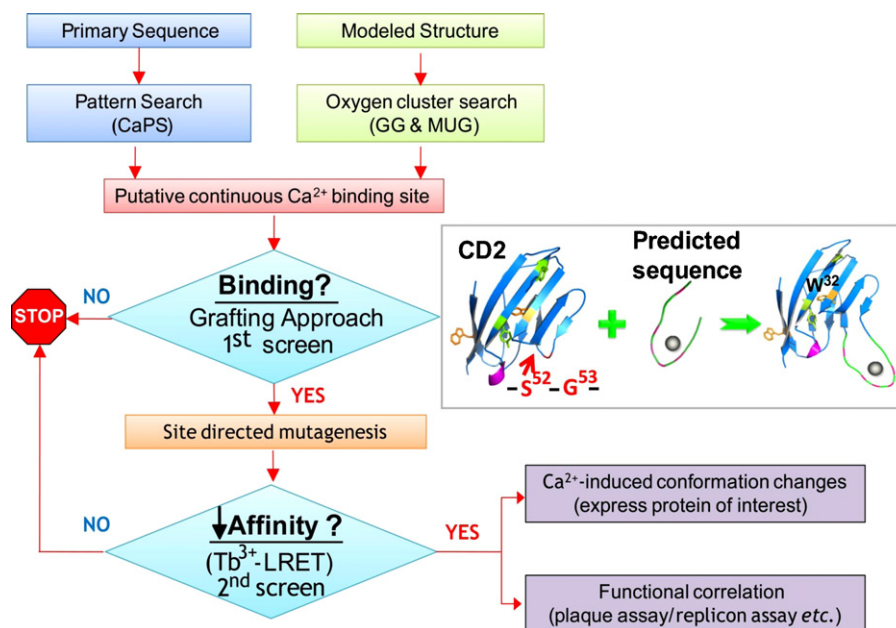
Fig. 5. Experimental approaches to validate predicted continuous Ca^{2+}-binding motifs and to correlate its biological relevance. Continuous Ca^{2+}-binding sites can be predicted either from the primary sequence by using the program CaPS or from 3D modeled structure by using GG and MUG algorithm (available at http://chemistry.gsu.edu/faculty/Yang/Calciomics.htm). As first screen, the predicted Ca^{2+}-binding sequences are inserted into a scaffold protein CD2 using the grafting approach. This approach allows one to conveniently monitor the metal binding process with fluorescence spectroscopy by taking advantage of the aromatic residue (W32 in CD2) sensitized Tb^{3+} luminescence resonance transfer (Tb^{3+}-LRET). Ca^{2+} competition assay can be further performed based on Tb^{3+}-LRET to obtain Ca^{2+}-binding affinity. If metal binds to the engineered protein, mutations on the proposed ligand residues will be subsequently introduced to double confirm the binding event. If the mutagenesis results in a decrease in binding, the predicted site has a high chance of binding Ca^{2+}. Possible Ca^{2+}-induced conformational changes will be examined by expressing the protein of interest. In addition, the functional correlation of Ca^{2+} binding can be followed by comparing the phenotypes of WT and mutant viruses.

in Supplementary Table 3) in proteins encoded by the genomes of almost 80 different viruses, covering the majority of virus families. In contrast to EF-hand motifs in cellular proteins, almost all of these predictions were single EF-hand motifs. The exceptions were putative dual EF-hand or EF-hand-like motifs in the envelope protein of HIV-1 and the immediate-early protein RSP40 of pseudorabies virus (a Herpesvirus). These putative EF-hand motif-containing proteins are involved in a wide range of viral processes, including attachment and entry (neuraminidase of Influenza A virus, Sendai virus and human parainfluenza virus 1; envelope glycoprotein of HIV-1; spike protein of rat coronavirus, murine hepatitis virus and bovine ephemeral fever virus; glycoprotein B of feline herpersvirus 1), virion structure (coat protein of beet yellow stunt virus, papaya ringspot virus and African horse sickness virus), precursor protein processing (nonstructural protease of rubella virus), nucleic acid modification and replication (mRNA-capping enzyme of alphavirus; RNA-dependent RNA polymerases of tobamovirus, respiratory syncytial virus, and influenza A virus; DNA methylase of sulfolobus virus; DNA polymerase of nucleopolyherosis virus and human herpesvirus 2), and transcriptional regulators (ICP0 of bovine herpesvirus 1; IE63 of human herpesvirus 3; ICP4 of equine herpesvirus 1). In addition, the functions of almost 20% of the proteins with predicted Ca^{2+}-binding motifs remain uncharacterized.

Subsequent to prediction of EF-hand and EF-hand-like Ca^{2+} motifs in viral proteins, experimental validation is required (Fig. 5). As a model, we used the putative EF-hand motif (*DASPD*G*TGDPLD*) predicted in the rubella virus nonstructural (NS) protease. Rubella virus (RUBV) is a positive-polarity, single-strand RNA virus in the *Togavirus* family. The NS protease, a papain-like cysteine protease with a Cys-His catalytic dyad, is a domain in a nonstructural polyprotein which functions to replicate the virus RNA through use of a complementary negative polarity RNA in the cytoplasm of infected cells [131]. The NS protease makes a single cleavage within the initial polyprotein precursor (∼2200 aa) to produce two prod-

ucts (∼1300 and 900 aa). In addition to being large polyproteins, the RUBV nonstructural proteins are only made in extremely small amounts, necessitating expression in heterologous systems for analysis. We therefore used two approaches: bacterially expressing the region of the NS protease containing the EF-hand motif as a minidomain and grafting the putative EF-hand motif itself into a CD2 scaffold protein [130]. Both approaches succeeded in showing that this putative canonical EF-hand motif specifically binds Ca^{2+} but not Mg^{2+} and monovalent cations [130]. The Ca^{2+}-binding affinity of this putative EF-hand was determined to be ∼200–300 μM, which agrees with the Ca^{2+} concentration of late endosomes and lysosomes (400–600 μM) [132,133], where the RUBV replication complex forms and replication occurs. The observed weak affinity is likely due to the presence of an Asp at the bidentate position 12 of the loop rather than a Glu, which ensures a high Ca^{2+}-binding affinity due to its larger side chain and stronger interaction with Ca^{2+}. Indeed, substitution of Glu at position 12 in CaM with Lys or Gln reduces Ca^{2+}-binding affinity by 10–100-fold [134]. Mutants (D-to-A mutations) of two of the predicted ligands at positions 5 and 12 of the loop were made in the CD2-grafted EF-hand motif and found to have lost the ability to bind Ca^{2+}. When these mutations were introduced into the virus, NS protease activity was rendered temperature sensitive, indicating that the function of Ca^{2+} binding was in stabilizing the protease [130]. While several viral proteases have been shown to bind Zn^{2+} as a stabilizing factor (including the RUBV NS protease), this was the first demonstration of Ca^{2+} binding by a viral protease. These approaches can be employed to validate predicted EF-hand and EF-hand-like Ca^{2+} motifs in other virus proteins.

4. Ca^{2+}-dependent virus–host interactions

Compared to the scarcity of reported viral CaBP's, host cells contain abundant CaBP's. Accordingly, viral proteins utilize a number of important cellular CaBP's, including proteins in the cytoplasm (e.g., annexin, calmodulin and S100), endoplasmic reticulum (e.g.,

Table 1
Interactions between cellular Ca^{2+}-binding proteins (CaBPs) and virus.

Cellular CaBP	Viral molecular identity	Virus	Consequences of interaction	Reference
Annexin II	p55^{GAG}	HIV-1	Facilitates virus entry and fusion in macrophages	[136]
	Glycoprotein B	CMV	Enhances binding and fusion to membranes	[137,168]
Annexin V	Small HBsAg	HBV	Participates in initial steps of HBV infection	[168]
Calmodulin	Nef	HIV-1	Alters T lymphocyte signaling pathway	[142,143]
	gp 160/gp41	HIV-1	Disrupts CaM signaling pathway	[146]
	gp 41	SIV		[147,169]
	p17^{GAG}	HIV-1		[148]
Calreticulin/calnexin	E1 and E2	RUB	Regulates viral glycoprotein maturation	[153]
	Viral RNA	RUB		[156]
	MP	TMV	Regulates cell-to-cell virus movement	[152]
	F, HN	SeV	Mediates maturation of glycoproteins	[158]
	gp160	HIV-1	Facilitates protein maturation	[159]
	P12^I	HTLV-1		[13]
	Tax	HTLV-1	Facilitates viral protein folding; possibly mediates the interaction with MHCI	[160]
ERC-55	E6	HPV		[165]
Fibulin-1	E6	HPV	Regulates cell migration and invasion	[167]
S100A10 (p11)	pol	HBV	Inhibits viral replication	[150]
	NS3	BTV	Mediates nonlytic virus release	[170]

Abbreviations: HIV, human immunodeficiency virus; CMV, cytomegalovirus; HBV, hepatitis B virus; SIV, simian immunodeficiency virus; RUB, rubella virus; SeV, Sendai virus; TMV, tobacco mosaic virus; HTLV, human T-cell lymphotropic virus; HPV, human papillomavirus; EBV, Epstein-Barr virus; BTV, bluetongue virus.

ERC-55, calreticulin, and calnexin), and extracellular matrix (e.g., fibulin-1) in their replication cycles (listed in Table 1).

4.1. Annexins

Annexin II, traditionally thought of as Ca^{2+}-dependent phospholipid-binding proteins expressed in monocytes, microglia and macrophages, has been implicated as functioning in membrane trafficking, endosome formation and vesicle aggregation [135]. It has been shown that the HIV capsid polyprotein precursor p55^{GAG} interacts with annexin II in macrophages. However, the Gag-annexin II interaction is only seen in productively infected macrophages but not in infected cells in which the virus is quiescent. Depletion of annexin II results in destabilization of lipid rafts and ablation of HIV virion assembly. In addition, annexin II was reported to promote HIV entry into macrophages through its interaction with phosphatidylserine in viral particles [136].

Direct interaction between annexin II and viral glycoproteins is also observed during CMV infection. The binding of annexin II to the CMV glycoprotein B (gpUL55) appears to be a Ca^{2+}-dependent process [137]. Similar to its interaction with HIV capsid polyprotein precursor p55^{GAG}, annexin II enhances the binding and fusion of CMV virus particles to phospholipid membranes, thereby maximizing its infectivity by promoting virus entry [138].

In addition, human liver annexin V, a Ca^{2+}-dependent phospholipid-binding protein present on the plasma membrane of human hepatocytes has been shown to interact with one of the forms of the HBV surface glycoprotein, the HBsAg, with a dissociation constant of 1.7 nM in the presence of Ca^{2+} [139].

4.2. Intracellular Ca^{2+} sensor protein—calmodulin

Calmodulin (CaM) is a small (148 amino acids), acidic (pI: ~4.0) CaBP that is ubiquitously expressed in eukaryotic cells. It consists of two globular and autonomous domains, each of which contains two EF-hand motifs. Through its binding to Ca^{2+} and the concomitant conformational changes that result, CaM is capable of transducing the intracellular Ca^{2+} signal changes into divergent cellular events by binding to an array of cellular proteins [140,141]. Two HIV proteins, Nef, an accessory protein, and gp160, the glycoprotein precursor, have been shown to interact with CaM in a Ca^{2+}-dependent fashion. Nef is a myristoylated protein expressed early in infection.

Nef has been shown to downregulate both CD4 and MHC-Class I cell surface receptors, both important in the cell mediated response, and to alter T lymphocyte signaling pathways. The latter effect is partially associated with its ability to strongly interact with CaM (with an apparent dissociation constant of 13.7 nM) [142]. The CaM-targeting sequence in Nef is contained within a 20-amino-acid basic domain at the N-terminus that shares a high degree of similarity with myristoylated neuron-specific protein kinase C substrate, NAP-22 [142,143]. Besides Nef, both the precursor gp160/gp41 has been shown to interact with CaM [144,145]. Two C-terminal CaM-targeting sequences in gp41 are shown to bind to CaM with dissociation constants of 31–41 nM [146]. Such interaction is speculated to disrupt the anti-apoptotic CaM signaling pathway by either reducing the amount of free cytosolic CaM or changing its subcellular localization [146]. The similar CaM-targeting sequence is also detected in simian immunodeficiency virus gp41 [147]. Adding more complexity to the scenario, the Gag gene products of HIV has been shown to interact with CaM [148]. The N terminus of p17 contains two contiguous CaM-binding sites, each of which binds CaM with affinities of ~10^9 M. In view of the diverse roles of Ca^{2+}/CaM-dependent signaling pathways, the interaction between all these HIV proteins with CaM is expected to play multiple roles to fit the HIV life cycle in response to altered Ca^{2+} signals.

4.3. Intracellular Ca^{2+} effector protein—S100A10

Bluetongue virus (BTV) is a double-stranded RNA arthropod-borne virus of the genus *Orbivirus* in the reoviridae family that causes catarrhal fever, a disease in ruminants of economical importance. The BTV nonstructural protein NS3 has been shown to interact with a host cellular protein S100A10 or p11, which harbors an N-terminal pseudo EF-hand and a C-terminal canonical EF-hand Ca^{2+}-binding motif. Two molecules of p11 serve as light chain components along with two heavy chains composed of annexin II to form the tetrameric calpactin complex. The calpactin complex is closely associated with Ca^{2+}-dependent exocytosis and secretory pathways [149]. Since NS3 also interacts with the outermost core protein (VP2) of assembled virus particles, the BTV NS3 is proposed to bring the virions together with the calpactin complex via p11 and further direct the newly assembled virus particles to the cellular exocytic machinery in infected cells. Use of this cellular exocyto-

sis pathway allows for nonlytic release of virus particles assembled within the infected cell.

P11 has also been demonstrated to interact with the polymerase (Pol) protein of HBV [150]. The HBV Pol protein plays multiple roles in virus replication by signaling the encapsidation and degradation of pgRNA, the RNA intermediate in the replication pathway, priming reverse transcription and catalyzing both RNA- and DNA-dependent DNA synthesis [151]. The p11 protein is capable of recruiting HBV Pol to PML nuclear bodies, a region that is of vital importance for cell proliferation, differentiation and anti-viral responses. This interaction is affected by intracellular Ca^{2+} concentration. Treatment of cells with valinomycin, a drug that promotes Ca^{2+} influx, leads to a decrease of p11 in the nuclear bodies, whereas the blocking of Ca^{2+} influx by EGTA results in a marked increase of Pol-p11 complex in the nuclear bodies. Thus, it is expected that the virus would favor a higher cytosolic Ca^{2+} environment to avoid host anti-viral activities. In this regard, another HBV protein, the X protein, induces an increase in the cytosolic Ca^{2+} concentration by reducing mitochondrial Ca^{2+} uptake and impairs the activity of plasma membrane Ca^{2+}-ATPase [31].

4.4. Ca^{2+}-binding ER chaperones—calnexin and calreticulin

Tobacco mosaic virus (TMV) is a positive-sense, single-stranded RNA virus that infects tobacco and other members of the plant family *Solanaceae*. The TMV cell-to-cell spread is mediated by the movement protein (MP), which is responsible for the transport of the viral genomic RNA through plasmodesmata. Calreticulin has been implicated in this process through its interaction with MP [152]. Overexpression of calreticulin interferes with the normal targeting of MP and delays the cell-to-cell movement.

Rubella virus (RUBV), discussed above in reference to an EF-hand Ca^{2+}-binding motif in its NS protease, is a positive-sense single-stranded RNA virus of the *Togaviridae* family. In addition to the nonstructural ORF containing the above-mentioned protease, the RUBV genome has a second ORF which encodes its virion structural proteins, capsid and glycoproteins E1 and E2. Both E1 and E2 have been demonstrated to interact with two Ca^{2+}-binding ER chaperones, calnexin and calreticulin [153]. Calnexin is a membrane-bound Ca^{2+}-binding ER chaperone that retains incorrectly or incompletely folded proteins [154]. Calreticulin consists of three domains, a globular N domain, a P domain rich in proline residues, and an acidic C-terminal tail domain. The N and P domains are responsible for the chaperone function and the highly acidic C-terminal tail is involved in buffering Ca^{2+} storage with high capacity (20–30 mol Ca^{2+}/mol protein) [155]. Calreticulin functions as a molecular chaperone in facilitating the folding of a number of proteins in the ER. Its activity is influenced by changes in the ER, such as the concentration of Ca^{2+} and ATP. During posttranslational modification, virtually all glycoproteins associate with these two chaperones, which work in concert to supply "quality control" in secretory protein maturation by promoting folding and oligomerization and suppressing degradation. By differentially interacting with RUBV E1 and E2, calnexin and calreticulin control the transport of these two glycoproteins. Since these glycoproteins associate with calnexin and calreticulin over a prolonged duration (>60 min), the maturation of RUBV glycoproteins has been proposed to be the rate limiting step of the transport of glycoproteins from ER to Golgi complex, where virion formation by budding occurs [153]. In addition, a stem-and-loop structure at the 3′ end of the RUBV genomic RNA was found to interact with the N-terminal domain of calreticulin [156]. While this interaction was hypothesized to (1) promote viral RNA translation and/or replication or (2) compartmentalizing the RNA to escape the surveillance by the host innate immune system, its biological significance remains in doubt [157].

In addition to positive-stranded viruses, viral proteins encoded by negative-stranded RNA virus, such as Sendai virus that belongs to the *paramyxoviridae* family, also interact with these two ER chaperones. Two glycoproteins on the surface of the enveloped Sendai virus particle, the fusion protein (F) and the hemagglutinin-neuraminidase (HN), mediate virus entry into host cells. Following their synthesis in infected cells, both proteins undergo maturation by interacting with different chaperones during their transport through ER and Golgi complex to the cell surface, the site of virus formation by budding [158]. F and HN have been reported to interact with ER chaperones with different kinetics. The F precursor protein, which is a type I integral membrane protein and can be cleaved in to two active subunits linked by disulfide-bonds, shows only transient interaction with calnexin ($t_{1/2} = 8$ min). The HN protein, a type II integral membrane protein that functions as a tetramer, has slower kinetics by sequentially interacting with Bip, calnexin and calreticulin to ensure the correct folding and assembly [158].

The newly synthesized HIV envelope glycoprotein gp160 has also been shown to interact with both calreticulin and calnexin to form a transient ternary structure, and thereby facilitate the folding and maturation of HIV glycoproteins [159]. The accessory protein, Tax, of HTLV-1 is primarily located in the nucleus and functions as a transcriptional transactivator. Nevertheless, the Tax protein can also be exported to the cytoplasm to interact with a number of host transcription factors including NF-κB. This nuclear transport process possibly involves the interaction of Tax with calreticulin [160]. Interestingly, calreticulin-mediated nuclear transport is dependent on Ca^{2+} [161]. It has been reported that Ca^{2+}-loaded calreticulin does not support nuclear export of leucine-rich NES-containing proteins. However, calreticulin restores the nuclear export capability after treatment with EGTA. It still remains to be tested whether this property has effects on the subcellular distribution of Tax. In addition to Tax, the viral protein P12^I, an ER/Golgi-residing protein encoded by the Px open reading frame of HTLV-1, has been shown to interact with both calreticulin and calnexin [162]. This interaction is thought to facilitate the folding of p12^I and to modulate the level of Ca^{2+} storage. Another possible role of such interaction is to retain calreticulin-MHC-I complexes in the ER or cis-Golgi, thereby blocking its association with β_2-microglobulin and the trafficking of the protein complex to the cell surface [163].

4.5. Hexa-EF-hand ER CaBP–ERC-55

Human papilloma viruses (HPV's) are small DNA viruses that cause epithelial lesions such as warts; some genital HPV's are associated with cervical cancer. The HPV E6 protein, a small polypeptide of approximately 150 amino acids, plays multiple roles in HPV infection. The HPV E6 has been found to possess oncogenic activity by stimulating immortalization of human keratinocytes and transforming established fibroblasts [164]. By targeting p53, Bak and Myc for degradation, E6 protein is capable of inhibiting apoptosis of infected cells. The HPV-16 E6 proteins also bind a hexa-EF-hand CaBP, ERC-55, that resides in the endoplasmic reticulum [165]. The interacting region in ERC-55 maps to a stretch of 25 amino acids in the fourth EF-hand motif that is capable of binding Ca^{2+} and folds into the typical helix-loop-helix conformation [166]. The exiting helix of the EF-hand motif, but not the Ca^{2+}-binding loop and the entering helix, is fully responsible for this interaction. The biological role of this interaction still remains uncertain.

4.6. Extracellular matrix CaBP–Fibulin-1

Yeast two-hybrid screening studies reveal that the E6 protein of oncogenic HPVs interacts with another CaBP, fibulin-1. Fibulin-1 is an extracellular matrix protein that has been implicated in cellular motility modulation, cellular transformation and tumorgenesis.

The E6 protein has been demonstrated to interact with fibulin-1 in COS-7 cells transiently transfected E6 from cancer-related HPV 16 or the transforming bovine papillomavirus type 1 (BPV-1) [167]. Interestingly, overexpression of fibulin-1 inhibits E6-mediated cellular transformation, suggesting that the fibulin-1 serves as a tumor suppressor. The two-hybrid assay further localizes the interacting region in fibulin-1 to amino acids 343–483, which covers an important region (amino acids 356–440) that is involved in the self-association, Ca^{2+}-binding and fibronectin-binding of fibulin-1. It has thus been postulated that the interaction between the oncogenic E6 protein and fibulin-1 disrupts these functions and inactivates the inhibitory effect of fibulin-1 on cell migration and invasion.

5. Conclusions

As we have summarized in this review, viruses take full advantage of the intracellular Ca^{2+} signaling network, from direct binding of Ca^{2+} by virus proteins to binding of virus proteins to cellular CaBP's to alteration of Ca^{2+} homeostasis. The remodeled Ca^{2+} network affects every stage of virus replication as well as cellular outcomes. The prediction by our algorithm of a large number of EF-hand-Ca^{2+}-binding motifs in a diverse collection of virus proteins further expands the repertoire of virus–Ca^{2+} interactions. The Ca^{2+} signaling field has been constantly reinvigorated with the continuous discovery of new components and expansion of the Ca^{2+}-signaling toolkits. The big challenge for the future will be to integrate all aspects of the Ca^{2+}–virus interplay to elucidate the entire picture of viral calciomics at molecular levels.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2009.05.005.

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