would contribute to the development of new therapeutic approaches for retrovirus-induced diseases.

2. Membrane Fusion by Retroviral Env Glycoprotein

Mechanism of membrane fusion by the retroviral TM proteins is described elsewhere in details [3–7] and is similar to those used by envelope proteins of other enveloped viruses [8, 9]. Briefly, the retroviral entry mechanism is proposed as follows. The TM protein is thought to have hairpin-like structure (Figure 1). The binding of SU with its cognate cell surface receptor induces conformational changes of the TM subunit. The N-terminal hydrophobic domain of the TM subunit called fusion peptide is exposed by the conformational change and inserted into host cell membrane. The TM protein then coverts to a trimer-of-hairpins conformation, and viral envelope and host cell membranes approach and mix. Finally, the fusion pore is formed and expanded to derive the viral core into host cell cytoplasm. This conformational change pathway of the TM protein induces the membrane fusion for the retroviral entry into host cells.

3. Retrovirus Receptors

In this section, we will mainly focus on the infection receptors for MLV and HIV, with which entry mechanisms are most extensively studied among retroviruses. Other reviews should be referred to concerning the infection receptors of animal retroviruses in general [10, 11]. MLVs are divided into four groups according to their host ranges and infection interference, and the four groups recognize different cell surface receptors. Ecotropic MLVs infect mouse and rat and bind to cationic amino acid transporter 1 (CAT1) as the infection receptor [12]. Amphotropic MLVs infect many types of mammals, and inorganic phosphate symporter 2 (Pit2) is the amphotropic infection receptor [13, 14]. Polytropic MLVs has a similar host range to the amphotropic MLVs. The amphotropic MLVs cannot infect amphotropic virus-infected cells, because Pit2 are already occupied by the amphotropic Env proteins, called infection interference. Whereas the polytropic MLVs can infect amphotropic virus-infected cells, indicating that the polytropic virus receptor is different from the amphotropic receptor. Polytropic MLVs recognize XPR1 for the infection [15–17], whose physiological function is unknown yet. Xenotropic MLVs recognize the XPR1 as polytropic MLVs, but do not infect mouse cells. These MLV infection receptors are all multimembrane spanning proteins.

The infection receptors of HIV are CD4 and one of chemokine receptors (CXCR4 or CCR5) [18]. However, HIV variants that do not require CD4 for the infection are sometimes isolated from AIDS patients [19, 20] though the infectivity of CD4-independent variants is much lower than that of CD4-dependent viruses [21]. Such CD4-independent HIV variants recognize multimembrane spanning CXCR4 or CCR5 as the sole infection receptor, like the MLVs.
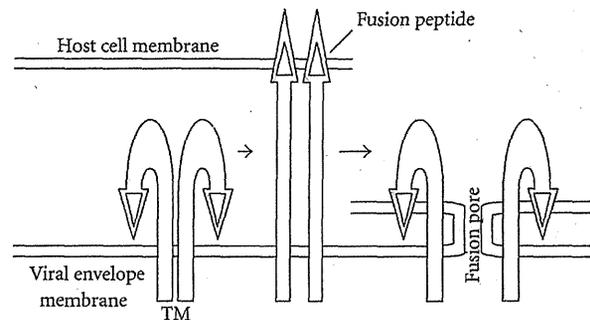

FIGURE 1: Conformational change of retroviral TM subunit for membrane fusion.

CD4 is a single-membrane spanning protein, and HIV variants recognizing CD4 as the sole infection receptor have not been isolated. CD4-independent variants of simian immunodeficiency virus (SIV) are more frequently isolated than CD4-independent HIV variants [22, 23]. It is thought that CD4-independent HIV variants are prototypes of CD4-dependent HIVs [22–24].

4. C-Terminal Tail of Retroviral Env Protein Inhibits Membrane Fusion

When retrovirus-producing and -susceptible cells are mixed, viral Env proteins on the cells can effectively interact with infection receptors on the neighboring susceptible cells via direct cell-to-cell contact. The interactions can have both positive and negative effects on the retrovirus replication. First, they can lead to cell-to-cell infection that allows very rapid and synchronized replication of virus compared to the cell-free infection [25, 26]. This can be advantageous for the virus replication in the presence of antiviral agents [27]. Second, the interactions can induce a negative effect, that is, the rapid apoptotic cell death, via syncytium formation [28–30]. This can be disadvantageous for the virus in that the sustained production of progeny virions becomes impossible. If the apoptotic cell death proceeded more efficiently than the virus replication, it eventually would result in poor progeny virus production. Therefore, it is conceivable that the retroviruses have some mechanisms to attenuate fusion capability of the envelope TM proteins in virus-producing cells and to primarily activate it in retroviral particles upon virion budding. Consistently, such mechanisms have been suggested for the Env TM proteins of MLV and HIV.

In the case of MLV Env protein, C-terminal 16-amino acid peptide of the TM subunit called R peptide is further cleaved by the retroviral protease after the budding [31, 32]. The R peptide-containing Env protein is expressed in the virus-producing cells. The R peptide-truncated MLV Env protein can induce syncytia in susceptible cells, but the R peptide-containing Env protein cannot, indicating that the R peptide negatively regulates the syncytium formation of virus-producing cells [33, 34]. Viral particles carrying the R peptide-containing Env protein have much lower infectivity

TABLE 1: Inhibitors used in studies of retroviral entry pathway.

Inhibitors	Target
Ammonium chloride	Acidification of intracellular vesicles
Bafilomycin A-1	Acidification of intracellular vesicles
Concanamycin A	Acidification of intracellular vesicles
Dynasore	Dynamin-dependent endocytosis
Chlorpromazine	Clathrin-dependent endocytosis
CA-074Me	Cathepsin B protease
Dynamin DN mutant[1]	Dynamin-dependent endocytosis
Caveolin DN mutant	Caveolin-dependent endocytosis
Clathrin DN mutant	Clathrin-dependent endocytosis
Eps 15 DN mutant	Endocytosis

[1] DN: dominant negative.

than those with the R peptide-cleaved Env, showing that the R peptide cleavage during virion maturation is required for the infectivity [35–37]. It has been reported that the R peptide controls the three-dimensional structure of the SU protein [38] and a disulfide bond between the SU and TM proteins [39], suggesting that the R peptide of TM subunit regulates the receptor-mediated SU conformational changes through the S–S bond between the SU and TM. It has been recently shown that the R peptide-cleaved TM forms separated Env legs, but the R peptide ties the TM legs together [40].

Although the C-terminal domain of the HIV TM protein is not cleaved, it is suggested that interaction between the HIV TM C-terminal region and Gag precursor protein suppresses the membrane fusion activity in virus-producing cells [41]. Processing of the HIV Gag precursor after budding abrogates the suppression of membrane fusion, and the mature virions gain sufficient fusion activity for the entry. The functions of C-terminal tails of retroviral Env proteins to inhibit membrane fusion are conserved among many retroviruses [42–45], though the mechanisms are different. The C-terminal domains of retroviral Env glycoproteins function to maintain the production of progeny virions by suppressing syncytium formation-directed apoptosis of virus-producing cells.

5. PH-Dependent Retrovirus Infection

Ammonium chloride, a weak base, neutralizes acid conditions in intracellular vesicles (Table 1). Concanamycin A and bafilomycin A-1 are specific inhibitors of the ATP-dependent proton pump/vacuolar ATPase (V-ATPase) that serves to acidify endocytic vesicles [46, 47]. To analyze the pH dependence of retrovirus entry, these compounds are frequently used. Additionally these inhibitors may affect trafficking of the intracellular vesicles, because siRNA-mediated knockdowns of subunits of V-ATPase complex affect trafficking of intracellular vesicles [48]. Previously it had been reported that ammonium chloride inhibits ecotropic MLV infection but does not amphotropic and xenotropic MLV infections, showing that ecotropic MLV infection occurs through acidic vesicles, but amphotropic and xenotropic MLV infections

do not [49, 50] (Table 2). The more specific inhibitors of endosome acidification (concanamycin A and bafilomycin A-1) suppress all of ecotropic, amphotropic, polytropic, and xenotropic MLV infections [51, 52]. At present, it is generally accepted that ecotropic MLV infection requires acidification, because all the studies consistently reported the suppression of ecotropic virus replication with the inhibitors of endosome acidification. In contrast, it has been shown that xenotropic MLV infections are not suppressed by bafilomycin A-1 [53] (Table 2). Due to the controversial results, the entry pathway of xenotropic MLV is not clear yet. Because different cell lines were used in those reports, the low pH requirement of the xenotropic MLV infection may be dependent on the used cell lines (see below).

In case of avian leukosis virus (ALV) infection, there are also several controversial reports. The earlier reports show that ammonium chloride and bafilomycin do not affect ALV infection, suggesting that ALV infection does not require the acidification [54, 55]. In contrast, it has been recently reported that lowering the pH results in quick and extensive cell-cell fusion by ALV [56] and that the acidification inhibitors suppress ALV infection [57, 58]. It is now thought that receptor binding of ALV induces the Env protein to convert to its prehairpin intermediate at neutral pH [59, 60], and then endosome acidification triggers the formation of the final fusion-active form of the Env protein [61–63]. It has been proposed that the discrepancy came from unusual stability of the Env prehairpin intermediate, consequent ability of fusion to proceed upon washout of the acidification inhibitors after several hours, and the relatively high pH requirement for the outer leaflet mixing [64]. Finally, it is considered that ALV entry requires endosome acidification.

The acidification inhibitors suppress infections by mouse mammary tumor virus (MMTV) [65], foamy virus [66], equine infectious anemia virus (EIAV) [67, 68], Jaagsiekte sheep retrovirus (JSRV) [69], and enzootic nasal tumor virus [70]. These results suggest that infections by many animal retroviruses are low pH dependent.

6. Internalization Pathways

The requirement of low pH for the retrovirus infections reveals that retrovirus particles are internalized into acidic intracellular compartments during virus replication. There are several different pathways for the internalization of molecules: (i) phagocytosis, (ii) macropinocytosis, (iii) clathrin- and dynamin-dependent endocytosis, (iv) caveolin- and dynamin-dependent endocytosis, (v) lipid raft- and dynamin-dependent endocytosis, (vi) clathrin-, caveolin-, and dynamin-independent endocytosis that requires lipid raft, and (vii) dynamin-, clathrin-, caveolin-, and lipid raft-independent endocytosis [48, 71]. Here we will briefly summarize the accepted mechanisms and roles of internalization, relevant to the present review [48, 72, 73].

6.1. Phagocytosis. Specialized cells such as macrophages, neutrophils, and monocytes clear debris and pathogens

TABLE 2: Differential dependence of HIV and MLV infections on endosome acidification.

Viruses	Dependence of acidification	Cell lines	Reference
Ecotropic MLV	Independent	Rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[49, 51, 52]
Amphotropic MLV	Independent	Mouse NIH3T3, rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[51, 52]
Polytropic MLV	Independent	Rat XC	[52]
	Dependent	Mouse NIH3T3, human RE671, rat XC	[52]
Xenotropic MLV	Independent	Human HT1080, HTX, porcine, rat XC	[49, 50, 52, 53]
	Dependent	Mouse NIH3T3, human RE671	[52]
CD4-dependent HIV	Independent	Human CEM, HeLa, C8166, VB	[49, 89–93]
	Independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dependent	Human 293T, HeLa, TE671	[21]

by phagocytosis. Signaling cascades induce the actin rearrangement and form membrane extensions that cover the target particles and engulf it. Phagosomes become acidic by fusion with lysosomes (pH 5.0-6.0). Debris internalized by phagocytosis is degraded in the acidic phagosomes (phagolysosomes).

6.2. Macropinocytosis.

Stimulation by certain growth factors or other signals causes membrane protrusions that fuse with the plasma membrane to form large intracellular vesicles known as macropinosomes that encapsulate large volumes of the extracellular fluid. Macropinosomes can either fuse with lysosomes (pH 5.0-6.0) or recycle back to the cell surface. There is no consensus as to the final fate of macropinosomes. Trafficking of macropinosomes seems to depend on cell type and mode of macropinocytosis induction.

6.3. Clathrin-Mediated Endocytosis.

After ligands bind to their receptors, the receptor proteins are internalized into intracellular vesicles called endosomes. The endosome formation requires dynamin GTPase, and the endosomes are coated by clathrin proteins. Many receptors are segregated from their ligands in early endosomes due to weakly acidic condition (pH 6.0). Early endosomes become more acidic by V-ATPase-mediated acidification (late endosomes/lysosomes) (pH 5.0-6.0), and separated ligands are degraded by endosome proteases. Certain receptors are transferred from early endosomes to recycling endosomes (pH 6.4) and are reused on the plasma membrane. Some proteins are also recycled from late endosomes/lysosomes through the trans-Golgi network. Lysosomes often form multivescular bodies.

6.4. Caveolin-Mediated Endocytosis.

Glycosylphosphatidylinositol (GPI)-anchored proteins, simian virus 40 (SV40), and cholera toxin trigger the formation of caveolae coated by caveolin proteins. These ligands are internalized into intracellular vesicles (pH 7.0) dependently on dynamin GTPase. The vesicles can be sorted to endosomes and become acidic.

6.5. Clathrin- and Caveolin-Independent Endocytosis.

Cholera toxin and SV40 can also be internalized via raft microdomains into GPI-anchored protein-enriched endosomes. Mechanisms regulating this internalization pathway are unclear as of yet.

7. Internalization of Retroviral Particles into Intracellular Vesicles

A dominant negative mutant of caveolin [74], siRNA-mediated knockdown of dynamin, and a dynamin inhibitor (dynasore) (Table 1) [52] suppress the amphotropic MLV infection, suggesting that amphotropic MLV particles are internalized by the dynamin- and caveolin-dependent endocytosis for productive infection (the fourth pathway). Ecotropic MLV particles are internalized into intracellular vesicles, but the vesicles are not colocalized with clathrin [75]. Furthermore, the dynamin-dominant negative mutant does not inhibit ecotropic MLV infection in human HeLa cells expressing the ecotropic MLV receptor, suggesting that ecotropic MLV particles are internalized by clathrin- and dynamin-independent endocytosis [75]. In contrast, another report indicates that siRNA-mediated knockdown of dynamin and dynasore suppresses ecotropic MLV infection in mouse NIH3T3, rat XC, and human TE671 cells expressing the ecotropic receptor [52] (Table 3). As mentioned above, the internalization pathway of ecotropic MLV might be dependent on the cell lines used. ALV [76] and EIAV [77] infections occur through clathrin-dependent endocytosis. JSRV infection required dynamin-dependent endocytosis [69]. Taken together, these reports strongly support a notion that infections by many animal retroviruses occur through endosomes and require endosome acidification.

All of intracellular vesicles do not necessarily become acidic. For example, macropinosomes can be recycled to plasma membrane before their acidification, and recycling endosomes are formed from early endosomes and are transferred to plasma membrane [48]. Because many retroviral infections require endosome acidification, if viral particles are internalized into recycling endosomes, infectivity would decrease. To prevent this, the interaction between retrovirus

TABLE 3: Differential internalization pathways of HIV and MLV infections.

Viruses	Internalization pathway	Cell lines	Reference
Ecotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Dynamin-, clathrin independent	Human HeLa	[75]
Amphotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Caveolin dependent	Mouse NIH3T3	[74]
Polytropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
Xenotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
CD4-dependent HIV	Dynamin dependent	Human HeLa	[95]
	Clathrin dependent	Human primary T lymphocyte	[95–97]
	Dynamin-, Eps15 dependent	Human HeLa	[98]
	Dynamin-, Eps15 independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dynamin-, Eps15 dependent	Human 293T, HeLa, TE671	[21]

Env proteins and the infection receptors is speculated to induce a signal to trigger the acidification of virion-containing intracellular vesicles.

8. Cleavage of Retroviral Env Proteins by Cathepsins

Many retrovirus infections require endosome acidification. Influenza virus infection also requires endosome acidification, and treatment of influenza virus particles with low pH buffer activates its membrane fusion, indicating that low pH treatment directly induces conformational change of the influenza virus hemagglutinin to the fusion-active form. In contrast, low pH treatment of MLV particles does not activate the membrane fusion. Why does ecotropic MLV entry require endosome acidification?

There is another mystery of the endosome-mediated infection. Proteins internalized into acidic late endosomes/lysosomes are generally degraded by endosome proteases including cathepsins. The acidification inhibitors suppress the degradation in late endosomes/lysosomes [47]. If the retroviral particles are degraded in late endosomes/lysosomes, the acidification inhibitors would enhance retrovirus infection. However, the acidification inhibitors rather suppress the infection [52]. Therefore, it is suggested that the retroviral particles incorporated into late endosomes/lysosomes are not degraded. Why are the retroviral particles not degraded in acidic late endosomes/lysosomes?

The finding that endosomal cathepsin proteases are necessary for the ecotropic MLV infection [78, 79] like Ebola virus infection [80] has provided a clue to understanding the questions. Because cathepsin proteases are activated by acidification, the ecotropic MLV entry into host cytoplasm requires cathepsin activation by acidification. The weakly acidic condition (pH 6) in early endosomes cannot activate cathepsin proteinases [81], suggesting that ecotropic MLV infection occurs via late endosomes/lysosomes. The acidification inhibitors suppress MLV infections by attenuating cathepsin protease activation. The evidence that the acidification inhibitors do not suppress the ecotropic MLV infection in active cathepsin-containing medium further
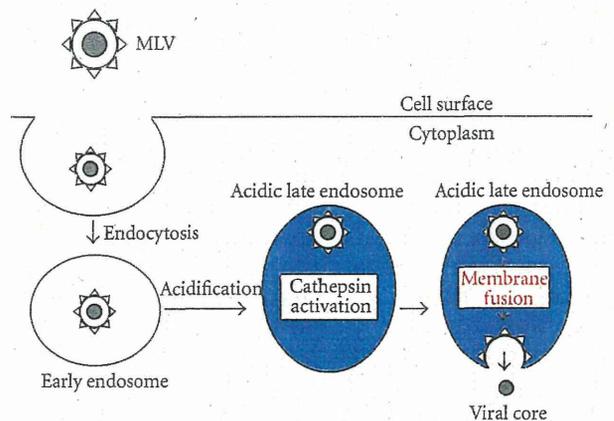

FIGURE 2: Entry pathway of ecotropic MLV in almost all susceptible cells. Blue area indicates acidic condition.

supports this conclusion [52]. Our current model for entry of ecotropic MLV is that cathepsin proteases digest MLV Env glycoproteins to generate fusion-active forms rather than to break them up completely, because treatment of ecotropic and amphotropic MLV particles with cathepsin B protease results in a few digested products of the Env proteins but not their disappearance [52, 79]. It is still unclear how the MLVs are not degraded in the late endosomes/lysosomes by other proteases.

In summary, the entry pathway of ecotropic MLV occurs as follows (Figure 2). Ecotropic MLV particles are internalized into endosomes, following the interaction of Env protein with the infection receptor. The viral particle-containing endosomes become acidic by V-ATPase. Cathepsin proteases are activated in the acidic late endosomes. The activated cathepsins cleave the ecotropic Env proteins to confer them fusion active. The cleaved Env proteins induce fusion between the viral envelope and host cell endosome membranes. Finally, the ecotropic MLV cores enter into host cytoplasm.

Although it is widely accepted that the ecotropic MLV infection requires endosome acidification and cathepsin proteases, the entry pathway of xenotropic MLV is not clear,

because of the contradictory reports [52, 53]. We have shown that xenotropic MLV infection requires endosome acidification and cathepsin proteases like the ecotropic MLV infection [52]. In sharp contrast, the Liu research group has reported that inhibitors of endosome acidification and cathepsin proteases do not inhibit the xenotropic MLV infection [53]. Different cell lines used in these studies may induce different entry pathways of the xenotropic MLV.

Unlike the ecotropic MLV entry, it has been reported that a low-pH pulse of JSRV particles overcomes the bafilomycin-mediated infection inhibition [69], EIAV infectivity is enhanced by low-pH treatment [67], and cell-cell fusion induced by the ALV Env protein is enhanced at low pH [55]. Additionally, analysis of the pH dependence of the foamy virus Env-mediated fusion in a cell-cell fusion assay revealed an induction of syncytium formation by a short exposure to acidic pH [66]. The low-pH treatment of these retroviruses may directly induce the conformational changes of their Env glycoproteins to fusion active forms without the proteolytic cleavage, like influenza virus.

9. PH-Independent MLV Infection in XC Cells

Although the acidification inhibitors attenuate the ecotropic MLV infection in almost all susceptible cells [49, 52], the inhibitors have no effect on the ecotropic MLV infection specifically in rat XC cells, suggesting that the ecotropic MLV infection in XC cells is independent of low pH [49] (Table 2). Furthermore, the R peptide-containing ecotropic Env protein can induce pH-independent syncytium formation in XC cells, but cannot in other susceptible cells [82, 83]. By these results, it had been widely thought that ecotropic MLV entry into XC cells occurs at cell surface membranes and does not require the internalization of virions into intracellular vesicles and acidification. This XC cell-specific pH-independent ecotropic MLV infection was one of the well-known mysteries in the MLV field [84, 85]. We found that a cathepsin inhibitor, CA-074Me, efficiently suppresses the ecotropic MLV infection in XC cells, like in other susceptible cells, suggesting that the ecotropic MLV infection in XC cells requires endosomal cathepsin proteases [52]. This result is inconsistent with the previous theory that the ecotropic MLV infection in XC cells does not occur through endosomes. Because the ecotropic MLV infection requires cathepsin proteases activated by endosome acidification, the acidification inhibitors would be proposed to suppress the MLV infection by attenuating cathepsin activation. However, the acidification inhibitors do not reduce cathepsin activity in XC cells, but do so in other cell lines, suggesting that cathepsin proteases are activated without endosome acidification in XC cells [52]. XC cells do not express so much cathepsin that activation is sufficient at suboptimal pH, because cathepsin activity of XC cells is comparable to that of NIH3T3 cells. These results prompted us to speculate that the ecotropic MLV infection in XC cells occurs through endosomes. The result that dynasore and siRNA-mediated knockdown of dynamin expression suppress the ecotropic MLV infection in XC cells strongly supports this hypothesis.
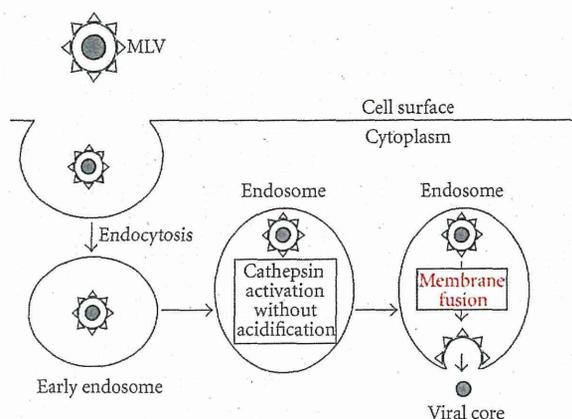

FIGURE 3: Entry pathway of ecotropic MLV in XC cells. Ecotropic MLV entry in XC cells may occur in acidic late endosomes, but endosome acidification is not required for the entry.

Taken together, the entry pathway of ecotropic MLV in XC cells is considered as follows (Figure 3). The ecotropic MLV particles are internalized into endosomes in XC cells, like in other susceptible cells. Cathepsin proteases are activated without endosome acidification. The activated cathepsins cleave the MLV Env protein, and the fusion between the viral envelope and host cell endosome membrane takes place for entry of the viral core into host cytoplasm. Because of the endosome acidification-independent activation of cathepsin proteases [52], the acidification inhibitors do not suppress the cathepsin protease activity and ecotropic MLV infection in XC cells. Additionally, this finding supports the above-mentioned hypothesis that the acidification inhibitors differentially affect retrovirus infections in different cell lines. The mechanism of acidification-independent cathepsin activation in XC cells is waiting to be resolved.

10. PH-Dependent Entry and PH-Independent Syncytium Formation by Retroviral Env Proteins

The R peptide-cleaved MLV Env protein induces the fusion between the viral envelope and host cell membranes for viral entry and syncytium formation in susceptible cells [33, 34]. Cells expressing the R peptide-truncated Env protein behave as large MLV particles and fuse with neighboring susceptible cells. Therefore, the syncytium formation by the retroviral Env proteins is thought to represent the membrane fusion in retroviral entry. Because the syncytium formation by the retroviral Env protein may contribute to the development of degenerative disorders like AIDS [28, 29], and because an endogenous retroviral Env protein (syncytin) induces syncytiotrophoblast formation [86], the elucidation of mechanism of retroviral Env-induced syncytium formation is essential to understand retroviral pathogenesis and placenta development. The MLV entry into host cells is dependent on low pH, but the syncytium formation by the R peptide-truncated Env protein is independent [33].

Furthermore, the viral envelopes fuse with host cell membrane in endosomes [52, 75], but the syncytium formation appears to result from the fusion of cell surface membranes of the Env-expressing and host cells. In addition, the Env glycoprotein of a CD4-independent HIV efficiently induces pH-independent syncytium formation [87], but infection by CD4-independent HIV occurs through acidic endosomes [21] (see below). Multiple interactions between the viral Env and infection receptor proteins in much larger areas of cell-cell contact than virus-cell contact may abrogate the requirement of endocytosis for the membrane fusion. The finding that a cell adhesion molecule, LFA-1, facilitates HIV-mediated syncytium formation but not HIV infection supports this idea [88]. If the syncytium formation by the Env protein is independent of endocytosis, cathepsin proteases would be unnecessary for the syncytium formation. However, cathepsin inhibitors suppress syncytium formation by the ecotropic MLV Env protein [79]. Secreted cathepsin proteases may be involved in the pH-independent syncytium formation by the Env protein. Further study is needed to understand the mechanism of pH-independent syncytium formation by the retroviral Env proteins.

FIGURE 4: Entry pathway of CD4-dependent HIV. Blue area indicates acidic condition.

11. Endocytic Pathway of CD4-Dependent and -Independent HIV Entry

There are many controversial reports of the role of endocytosis in CD4-dependent HIV infection [94] (Tables 2 and 3). Early reports indicate that the acidification inhibitors enhance [89–91] or do not affect CD4-dependent HIV infection [92, 93], suggesting that the HIV does not enter into host cells via acidic vesicles. However, recent reports show that dynasore and chlorpromazine attenuate CD4-dependent HIV infection [95–97]. In addition, dominant negative mutants of dynamin and Eps15 inhibit CD4-dependent HIV infection [98]. Furthermore, analysis of localization of labeled HIV particles revealed that the HIV particles are internalized into intracellular vesicles [95, 99–102]. It has been reported that envelopes of HIV particles fuse with host cell membranes in intracellular vesicles by the following observation [95]. Envelopes of HIV particles were labeled with a hydrophobic fluorescent compound. When fusion of the labeled HIV envelope with host cell membrane occurs, the fluorescent compound is diluted and the fluorescent signals disappear. The vanishing of the fluorescent signals was observed in the intracellular vesicles but not at cell surfaces. These results suggest that HIV entry into the host cell cytoplasm may occur via endosomes.

Interestingly, endosome acidification inhibitors attenuate infections by CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, suggesting that CD4-independent HIV entry may occur through acidic late endosomes, like many animal retroviruses [21]. The CD4-dependent HIVs can infect CD4-negative trophoblastic cells though the infection is 100 times less efficient than CD4-dependent Env-mediated infection [103]. HIV infection of trophoblasts forming the placental barrier may cause the mother-to-child transmission of HIV [104]. This infection
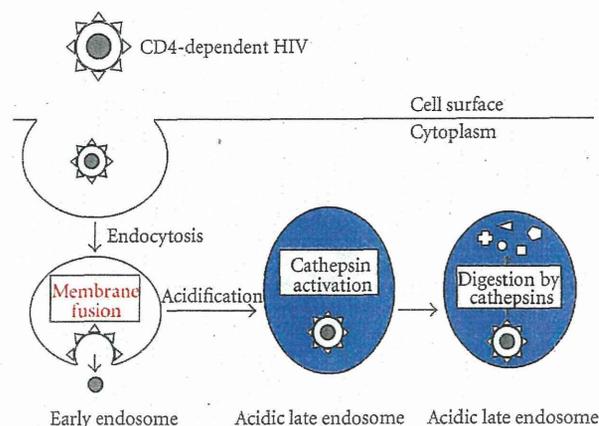
occurs through an unusual entry pathway that is clathrin-, caveolin-, and dynamin-independent endocytosis requiring free cholesterol [71].

12. Degradation of HIV Particles by Endosome Proteases

Because acidification inhibitors enhance CD4-dependent HIV infection [89–91], HIV entry is independent of low pH, and the viral particles internalized into acidic late endosomes are degraded [105]. In other words, a proportion of HIV particles are internalized into acidic late endosomes although the internalization into late endosomes is not associated with the HIV productive infection. Consistently, the HIV particles appear to be internalized into acidic compartments shortly after inoculation into host cells [100].

In summary, entry pathway of CD4-dependent HIV is considered as follows (Figure 4). The HIV particles are internalized into host cells by endocytosis, and the entry is independent of endosome acidification. HIV entry mainly occurs at early endosomes, and the HIV particles internalized into acidic late endosomes are degraded by endosome proteases.

It has been reported that a cathepsin inhibitor CA-074Me more significantly enhances CD4-independent HIV infection than CD4-dependent infection, and cathepsin protease activity in host cells is reverse-correlated with cellular susceptibility to the CD4-independent HIV infection [21]. These results suggest that CD4-independent HIV entry may occur at acidic late endosomes, and that viral entry competes with virion degradation by cathepsin proteases (Figure 5).

Degradation by endosomal proteases in acidic vesicles following phagocytosis/macropinocytosis/endocytosis functions as an innate immune reaction against microbes to digest them and generate antigen peptides presented to helper T cells on MHC class II [106]. In fact, the activation of toll-like receptor signaling by LPS enhances cathepsin expression [21]. The CD4-dependent HIVs might evolve
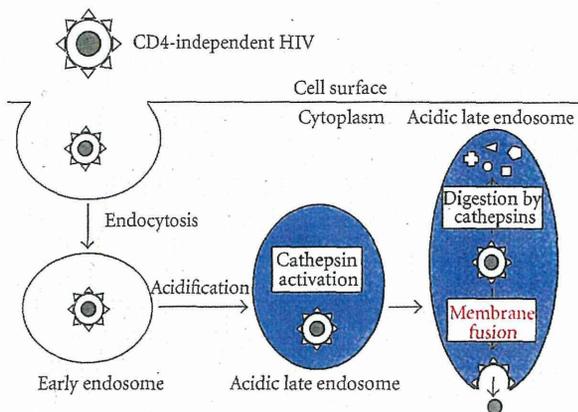
FIGURE 5: Entry pathway of CD4-independent HIV. Blue area indicates acidic condition.

from CD4-independent viruses to overcome the endosome protease-mediated immunity. Some microbes express cystatin-like cathepsin inhibitors to protect themselves from the cathepsin-mediated immunity [107, 108]. Instead of having a cathepsin inhibitor, the CD4-dependent HIVs might gain the acidification-independent entry mechanism to protect from the endosome protease-mediated immunity.

In contrast to the CD4-dependent HIV entry pathway, ecotropic MLVs utilize these cellular innate immune reactions of endocytosis, acidification, and digestion by endosome proteases to enter into the host cell cytoplasm. By the ecotropic virus entry mechanism, the viruses can escape from these host immune reactions. It is suggested that the CD4-dependent HIV entry utilizes endocytosis, but not acidification and proteolysis by endosome proteases. The CD4-dependent HIV particles may be degraded by endosome proteases in acidic endosomes, and the infection titer is reduced [89, 91]. The CD4-dependent HIV Env proteins indeed contain several amino acid motifs that are digested by cathepsins [109, 110]. The ecotropic MLVs also have cathepsin-recognized amino acid motifs, but the digestion may activate the membrane fusion capability of the Env protein.

As mentioned above, the cathepsin inhibitor enhances CD4-independent HIV infection in cells with relatively higher level of cathepsin protease activity [21]. While, treatment of such cells with CA-074Me at higher concentration attenuates the CD4-independent infection. In addition, CA-074Me suppresses the CD4-independent HIV infection in cells with lower cathepsin activity (unpublished data). These results suggest that cathepsin proteases are required for the CD4-independent infection. Therefore, Env glycoproteins of the CD4-independent HIVs may be digested by cathepsin proteases to a fusion-active form, like the ecotropic MLV Env protein. Consistently, cathepsin proteases enhance CD4-dependent HIV infection and confer CD4-negative cells susceptible to CD4-dependent HIV infection [111–113]. Cathepsin-mediated digestion of CD4-dependent HIV Env protein may induce membrane fusion without CD4 binding.

HIV particles in acidic endosomes are degraded by many endosome proteases including cathepsins. However, when the HIV Env proteins are digested only by a cathepsin, the infectivity may be enhanced.

13. Entry of Targeted Retroviral Vector

Retroviral vectors are valuable tools in molecular biology research and human gene therapy. Several fundamental properties of retroviral vectors remain to be improved for effective gene transfer to specific target cells [114]. The effectiveness will be greatly enhanced, if their infection tropism is artificially modified to target specific cells [115]. There have been various attempts to establish redirecting infection tropism by genetically incorporating heterogenous ligands into the retroviral Env proteins [116–121]. However, retroviral vectors containing such modified Env proteins suffer from very low transduction efficiency or are not infectious. The redirected transductions of retroviral vectors with chimeric Env proteins are enhanced by the endosome acidification inhibitors, suggesting that the targeted vector particles internalized into acidic endosomes are degraded by endosome proteases [120, 122].

Retroviral vectors carrying the ecotropic Env proteins chimeric with SDF-1α [123] and somatostatin [124] can transduce cells expressing CXCR4 and somatostatin receptor, respectively, as efficiently as retroviral vectors with the wild-type Env protein. It has not been examined whether efficient infections by the redirected retrovirus vectors occur through endosomes. Because the SDF-1α-chimeric Env protein appears to induce infection by the same mechanism as the wild-type Env protein [125], the redirected infection may occur through endosomes and require endosome acidification, like the wild type MLV Env protein. Elucidation of the entry pathways of these targeted retroviruses will likely contribute to the development of efficient cell lineage-specific retrovirus vectors.

14. Endocytic Entry of Ebola Virus-Pseudotyped Retrovirus Vector

Retrovirus vectors can be pseudotyped with glycoproteins of various enveloped viruses. The pseudotyped retrovirus vectors enter into host cells by the entry mechanisms of the heterologous viral glycoproteins. Because the retrovirus vectors do not produce replication-competent viruses and the protocol is relatively simple, pseudotyped retrovirus vectors are widely used to identify entry pathways of various enveloped viruses [126–128].

A dominant negative mutant of Eps15, siRNA-mediated knockdown of clathrin, and chlorpromazine suppress infection by an HIV vector pseudotyped with Ebola virus glycoprotein (GP), indicating that Ebola virus GP-mediated entry occurs through clathrin-dependent endocytosis [129]. Virion morphologies of the pseudotyped HIV vector and Ebola virus are much different. The pseudotyped HIV vector particles are round and the diameter is around 100 nm regardless of viral envelope glycoproteins. Whereas Ebola
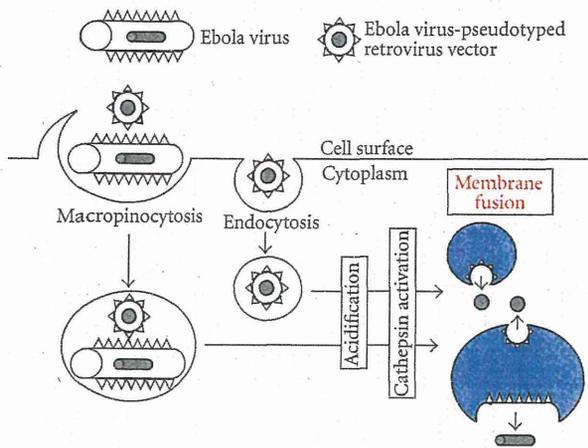
FIGURE 6: Entry pathways of Ebola virus and Ebola virus-pseudotyped retrovirus vector. Blue area indicates acidic condition.
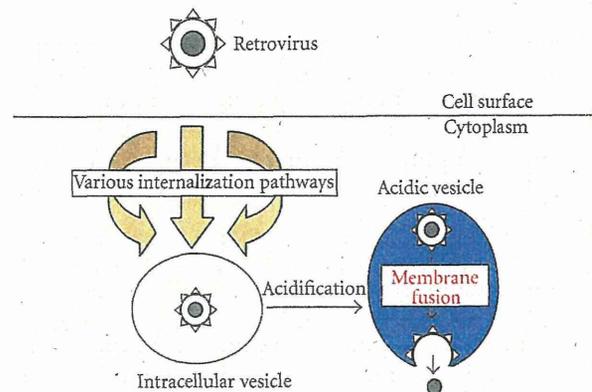

FIGURE 7: Retrovirus particles are internalized into intracellular vesicles by various pathways, and vesicle acidification is necessary for the infections.

virus virions are long and filamentous as the name of filovirus should show. Typical clathrin-coated vesicles are large enough to incorporate the HIV vector particles, but not Ebola virus particles. Therefore, Ebola virus particles cannot be internalized into the endosomes. Does Ebola virus enter into host cells through endosomes? The finding that Ebola virus entry occurs via macropinosomes resolved this problem [130–133] (Figure 6). Macropinosomes have enough size to incorporate Ebola virus particles. However, entry of intact Ebola virus is still dependent on dynamin, which is not involved in classical macropinocytosis [133], and is partially inhibited by inhibitors of clathrin-dependent endocytosis [132]. In addition, it has been reported that the Ebola virus entry through macropinocytosis or endocytosis is dependent on the cell lines used [134]. Therefore, the entry route of Ebola virus is not clear yet. The Ebola virus infections via endocytosis and macropinocytosis both require acidification and cathepsin proteases [80, 135]. Although the pseudotyped retrovirus vector is useful to study the entry mechanism of viral envelope proteins, we should notice the possibility that entry pathway of the pseudotyped retrovirus vector is different from that of the original virus.

Size of macropinosomes is enough to incorporate not only Ebola virus particles but also pseudotyped HIV vector particles. Therefore, Ebola virus-pseudotyped HIV vector entry can occur through macropinocytosis (Figure 6). There is a report showing that HIV infection occurs through macropinosomes [102]. If host cells have both dynamin-independent macropinocytosis and -dependent endocytosis, the inhibition of dynamin function does not significantly affect the pseudotyped HIV vector infection. If host cells have endocytosis but not macropinocytosis, the inhibition of dynamin function severely suppresses the pseudotyped HIV vector infection. Retrovirus entry may be able to occur through several distinct internalization pathways for productive infection (Figure 7). This may be the reason why the inhibitors differentially affect retrovirus infections in different cells. Pathways of retrovirus internalization into

intracellular vesicles may be unimportant for the productive infection. The GP of Ebola virus that enters host cells via macropinosomes can use endocytosis for the productive entry, when the retrovirus vector is pseudotyped with the Ebola virus GP. This result strongly supports the idea.

15. Conclusion

Infections by many animal retroviruses occur through endosomes and require endosome acidification. The activation of cathepsin proteases by endosome acidification is required for ectopic MLV infection. Whereas acidification directly induces conformational changes of several retroviral Env proteins to the fusion active forms. There are several internalization pathways of retrovirus particles, and the viral internalization pathways appear to be different in different cell lines. CD4-independent HIV infection may occur through endosomes and require endosome acidification, like other animal retroviruses. CD4-dependent HIV infection is thought to occur through endosomes but does not require endosome acidification. The CD4-dependent and -independent HIV particles are both degraded by endosome proteases, when the viral particles are internalized into acidic late endosomes. Retrovirus vectors pseudotyped with other viral envelope proteins are widely used to understand the entry mechanisms of the envelope proteins. However, entry pathway(s) of the pseudotyped retroviral vector could be different from that of the original virus.

Retroviruses require cellular biological events of internalization, vesicle acidification, and cathepsin proteolysis for their entry into host cells. These biological events, especially in phagocytosis, function to protect host cells from microbe infection. Retroviruses utilize these immune reactions to enter into host cells. This entry mechanism of retroviruses is the best strategy to overcome the host immune attack, and many viruses other than retroviruses also enter into host cells by similar mechanisms [72, 136].

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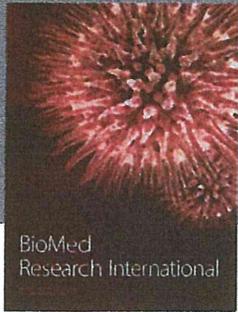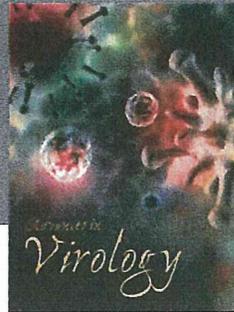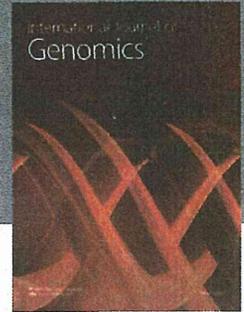
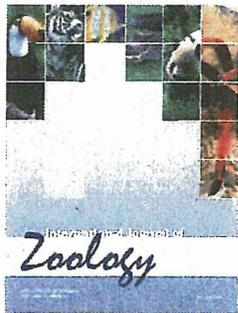BioMed
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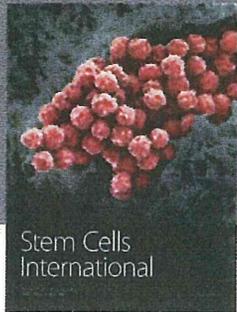
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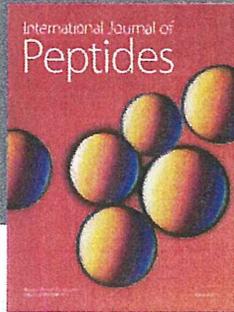
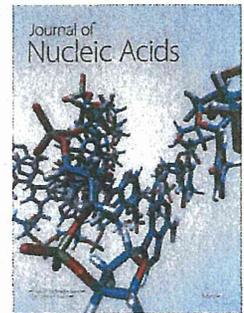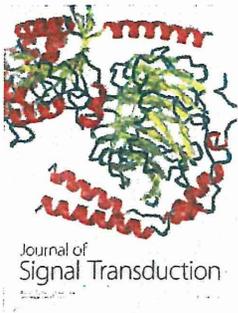International Journal of
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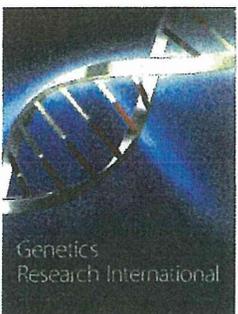
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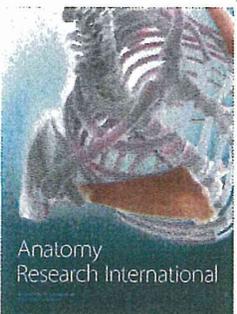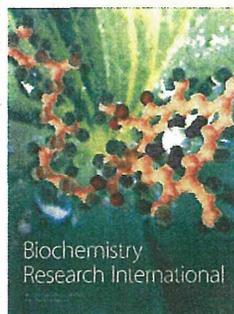http://www.hindawi.com
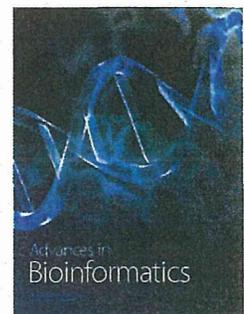
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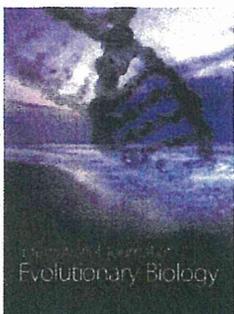
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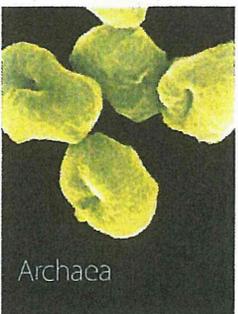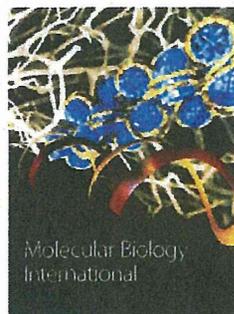
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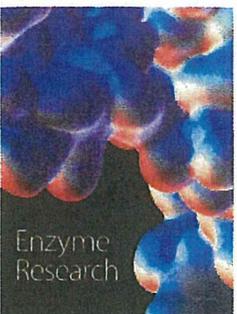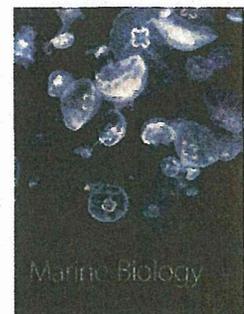
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Serum Starvation Activates NF-κB Through G Protein β2 Subunit-Mediated Signal

Tomoko Kohno, Yoshinao Kubo, Kiyoshi Yasui, Megumi Haraguchi, Sayuri Shigematsu,
Koon Jiew Chua, Toshifumi Matsuyama, and Hideki Hayashi

Several cell stresses induce nuclear factor-kappaB (NF-κB) activation, which include irradiation, oxidation, and UV. Interestingly, serum-starving stress-induced NF-κB activation in COS cells, but not in COS-A717 cells. COS-A717 is a mutant cell line of COS cells that is defective of the NF-κB signaling pathway. We isolated genes with compensating activity for the NF-κB pathway and one gene encoded the G protein β2 (Gβ2). Gβ2 is one of the G protein-coupled receptor signaling effectors. In COS-A717 cells, Gβ2 expression is significantly reduced. In Gβ2 cDNA-transfected COS-A717 cells, the NF-κB activity was increased along with the recovery of Gβ2 expression. Furthermore, serum-starving stress induced the NF-κB activity in Gβ2-transfected COS-A717 cells. Consistently, the serum-starved COS cells with siRNA-reduced Gβ2 protein expression showed decreased NF-κB activity. These results indicate that Gβ2 is required for starvation-induced NF-κB activation and constitutive NF-κB activity. We propose that serum contains some molecule(s) that strongly inhibits NF-κB activation mediated through Gβ2 signaling.

Introduction

NUCLEAR FACTOR-KAPPAB (NF-κB) is a ubiquitously expressed transcription factor with critical roles in cell survival, proliferation, apoptosis, immune response, and inflammation. NF-κB usually exists as a heterodimer of p50 and p65 (Rel A), and is kept in the cytoplasm through an association with inhibitor of kappaB (IκB) inhibitory proteins. After various stimulations, the serine residues at positions 32 (S32) and 36 (S36) in the IκB proteinare phosphorylated (Brown et al., 1995) by the IκB kinase (IKK) complex (Zandi et al., 1997), and the IκB protein is degraded by the ubiquitin–proteasome pathway (Chen et al., 1995). The IKK complex consists of two catalytic subunits, IKK1 and IKK2 (also referred to as IKKα and IKKβ), and a regulatory subunit, NEMO (Yamaoka et al., 1998). Cytokines and various cell stresses, including irradiation (Criswell et al., 2003), oxidation (Marshall et al., 2000), and UV (Kato et al., 2003), induce NF-κB activation. Serum starvation also activates NF-κB in various cell lines (Ryter and Gomer, 1993; Grimm et al., 1996), indicating that serum contains unknown inhibitor(s) of NF-κB.

On the other hand, constitutively active NF-κB exists in certain normal cells (Pagliari et al., 2000; Lilienbaum and Israel, 2003) and several tumor cells without stimulation (Mori et al., 1999; Lind et al., 2001). However, the mechanism by which NF-κB is constitutively activated in these cells is not known. COS cells have a relatively high level of basal NF-κB activity. We established a mutant cell line, COS-A717, with a defective NF-κB signaling pathway (Kohno et al., 2008). The basal level of NF-κB activity in the COS-A717 cells was reduced by as much as sevenfold, as compared with that in the parental COS cells. Serum starvation induced NF-κB activation in the parental COS cell line, but not in the COS-A717 cell clone. Since the COS-A717 cell clone was constructed by the treatment of COS cells with a frameshift-inducing agent, it is most likely that the NF-κB activating factor(s) expressed in the parental COS cells is not functional in the COS-A717 cells. We previously isolated the B cell activating factor of the TNF family (BAFF) receptor as an NF-κB activator in COS-A717 cells (Kohno et al., 2008). However, the original COS cells do not express BAFF-R, indicating that BAFF-R is not responsible for the defective NF-κB signaling in the COS-A717 cells, and activates NF-κB through a salvage pathway.

In this study, we isolated the guanine nucleotide-binding protein β2 subunit (Gβ2) cDNA as another NF-κB activator by screening a human spleen cDNA expression library. The guanine nucleotide-binding proteins (G proteins) are signal transducers required for various G protein coupled receptor (GPCR)-effector networks (Xie et al., 2000; Wu et al., 2001; Albert and Robillard, 2002). GPCRs 'transduce signals through heteromeric G proteins, and several of them activate NF-κB (Xie et al., 2000; Grabiner et al., 2007; Sun et al., 2009). The heteromeric G proteins consist of α, β, and γ subunits,

and the α subunit has GTPase activity. When GPCRs interact with their ligands, the active GTP-bound α subunit is released from the heteromeric G protein complex, and Gα and Gβγ induce downstream signaling (Stephens et al., 1994). The Gβ2 expression level in the parental COS cells is much higher than that in the mutant COS-A717 cells. Transfection of a Gβ2 expression plasmid activated NF-κB in COS-A717 cells. The knockdown of Gβ2 expression by siRNA in COS and HT1080 cells reduced the basal NF-κB activity. These results indicate that the activation of the GPCR signal pathway by Gβ2 results in constitutive NF-κB activation in the transformed cells, and the defect of Gβ2 expression is one of the determinants for reduced NF-κB activity in the COS-A717 mutant cells.

Serum starvation activates NF-κB in COS cells, but not in COS-A717 cells. Transfection of COS-A717 cells with Gβ2 restored the starvation-induced NF-κB activation. These results show that NF-κB activation by serum starvation occurs through the Gβ2 signaling pathway, and the inhibitor(s) present in serum sppress the Gβ2 signal. Taken together, our findings suggest that the constitutive NF-κB activation in transformed cells is induced by the GPCR signaling pathway through Gβ2, and that serum contains factor(s) reducing NF-κB activity by suppressing the GPCR signaling.

Materials and Methods

Plasmids and reagents

The human spleen cDNA library was purchased from Life Technologies. The five-tandem κB luciferase reporter vector (5×κB luciferase) was purchased from Stratagene. The IκBα superrepressor (IκBα-SR) expression plasmid was described previously (Sugita et al., 2002). The expression vectors for the dominant negative forms of IKK1 (IKK1.DN), IKK2 (IKK2.DN), and NEMO (NEMO.DN) were kind gifts from Dr. Yamaoka (Tokyo Medical and Dental University, Tokyo, Japan) (Hironaka et al., 2004). The expression vector for the dominant negative mutant of Akt (Akt.DN) was generously provided by Dr. V. Stambolic (Ontario Cancer Institute, Toronto, Canada). Wortmannin was purchased from Sigma.

Cells

The mutant cell lines COS-A717 and COS-A717-GS were described previously (Kohno et al., 2008). The mouse spleen cells were obtained from a C57BL/6 mouse. COS (Kohno et al., 2008), HT1080 (Jones et al., 1975), and HEK293T cells were maintained in the Dulbecco's modified Eagle's medium, and ST1 cells (Yamada, 1996) were maintained in the RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere.

Expression cloning of Gβ2

Isolation of genes with compensating activity for the NF-κB activation pathway was performed according to the previously described method (Kohno et al., 2008). Briefly, COS-A717-GS cells were transfected with a human spleen cDNA library (Life Technologies) using the FuGene 6 reagent. After 48h of transfection, the top 0.5% fraction of fluorescent cells was collected using a FACStar Plus (Becton, Dickinson and Co.). Plasmids were extracted from sorted

cells (Hirt, 1967), amplified in bacteria, and used in three subsequent rounds of flow cytometry-based enrichment. Individual bacterial colonies obtained from the third sorting were grouped into pools of 50 colonies. Positive pools were subdivided further into subpools with half the number of colonies, and were subjected to repeated screening. This process finally yielded independent clones that conferred compensation for the NF-κB activation pathway in COS-A717 cells.

Transfection and luciferase assay

Cells were transfected with a 5×κB-luciferase reporter and a Gβ2 expression plasmid, as indicated in the text and figure legends. Transient transfections were performed using the FuGene 6 reagent (Roche). When necessary, additional DNA (pcDNA3.1) was added to equalize the amount of transfected DNA in each sample. At 48h post-transfection, the κB-directed expression of firefly luciferase was determined, using luciferase assay reagents (Promega), and the luciferase activities were measured with a BioOrbit 1254 luminometer. The relative transfection efficiency in each sample was determined by measuring the Renilla luciferase activity. The data were normalized per transfection efficiency. Data shown are averages and SD from three independent experiments.

Western blot analysis

Cell extracts were prepared from the cells transfected for the luciferase assay. Cell lysates were resolved by 12.5% SDS PAGE, transferred onto an Immobilon-P membrane (Millipore), and blocked with 5% nonfat dry milk in TBS with 0.5% Tween 20. The blots were incubated with anti-Gβ2, anti-IKK1, anti-IKK2, anti-NEMO, anti-Akt1, anti-IκBα, and anti-phosho S32, and S36-containing peptide of IκBα antibodies (Santa Cruz Biotechnology; refer to Tables 1 and 2), or an anti-β-actin antibody (Chemicon), followed by an incubation with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig (Amersham Pharmacia Biotech). The blots were visualized with the ECL detection system (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay

Preparation of nuclear extracts for electrophoretic mobility shift assays (EMSAs) was performed as described previously (Sugita et al., 2002). The consensus κB site 5'-AGTTGAGG GGACTTTCCCAGGC-3' and mutant 5'-AGTTGAGGCGAC TTTCCCAGGC-3' oligonucleotides were obtained from Santa Cruz Biotechnology. The double stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP, using T4 polynucleotide kinase (Takara). The reaction was conducted in a total volume of 10 μL, using 10 μg of nuclear extract, 1 μg of poly(dI-dC), 20mM HEPES-NaOH (pH 7.6), 100mM NaCl, 1mM DTT, 1mM PMSF, and 2% glycerol. The binding reaction mixture was incubated with 10,000cpm of radiolabeled probe for 15min. For the competition and supershift assays, a 20-fold excess of unlabeled or mutant oligonucleotide, and the antibodies to p65 or p50 (Santa Cruz Biotechnology) were added to the reaction, respectively. The samples were loaded onto a 5% nondenaturing polyacrylamide gel, which was run in a 0.5×TBE buffer. After

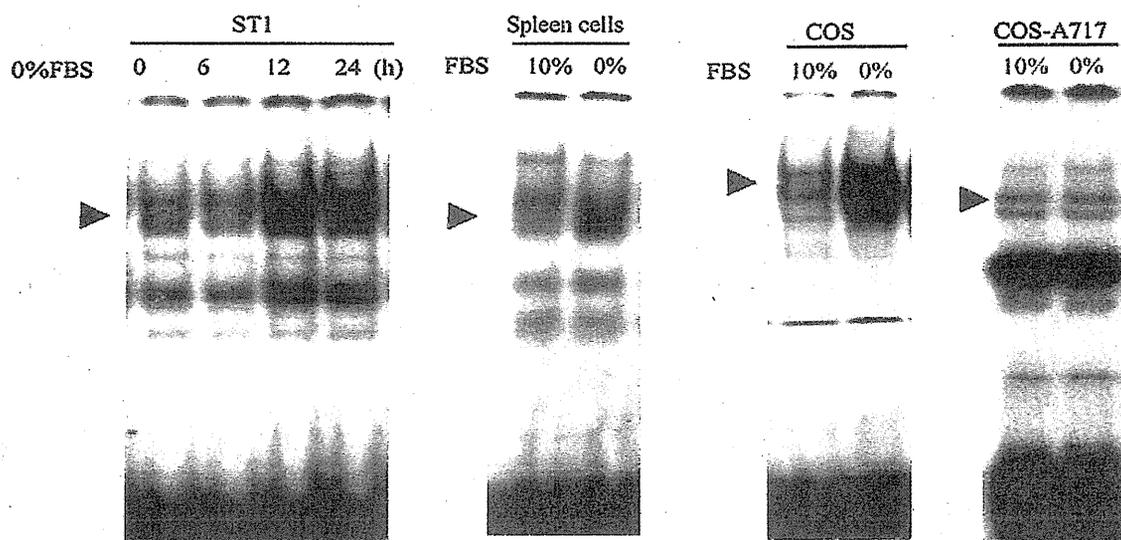.FIG. 1.   Serum-starving stress-induced nuclear factor-kappaB (NF-κB) activation. The nuclear extracts were incubated with a ³²P-labeled NF-κB.consensus oligonucleotide, and analyzed by an electrophoretic mobility shift assay. Nuclear cell extracts from ST1 cells, which were cultured without fetal bovine serum (FBS) for 0, 6, 12, 24 h (*ST1 panel*). The mouse spleen cells were cultured with 10% FBS or 0% FBS for 24 h (*Spleen cells panel*). Nuclear cell extracts from COS (*COS panel*) and COS-A717 (*COS-A717 panel*) cells, which were cultured with 10% FBS or 0% FBS for 24 h. The *arrowhead* indicates the NF-κB-containing complex.

electrophoresis, the gel was dried and processed for auto-radiography.

siRNA

The nucleotide sequences of the two siRNAs for Gβ2 are as follows:
#1 sense 5'-CAUCUGCUCCAUCUACAGCdTdT-3',
anti-sense 5'-GCUGUAGAUGGAGCAGAUGdTdT-3';
#2 sense 5'-AGACCUUCAUCGGCCAUGAdTdT-3',
anti-sense5'-UCAUGGCCGAUGAAGGUCUdGdT;

and sense 5'-GGCUACGUCCAGGAGCGCAdTdT-3', anti-sense 5'-UGCGCUCCUGGACGUAGCCdTdT-3' for GFP. The annealed oligonucleotides were transfected by using Lipofectamine 2000 (Invitrogen). Cells were maintained in the Dulbecco's modified Eagle's medium without FBS at the transfection. Cells were harvested after 24 and 48 h after the transfection for COS and HT1080 cells, respectively. For the luciferase assay, cells were transfected with the 5 × κB-luciferase reporter using the FuGene 6 reagent, at 6 h after the siRNA transfection.

Results

Serum-starving stress induces NF-κB activation

Cell stresses, such as irradiation, UV, and oxidation (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/dna), induce NF-κB activation. Cells are usually cultured with 10% FBS in medium *in vitro*. Serum includes various factors and nutrients for cell survival and proliferation, and thus serum starvation ceases cell proliferation, and then induces cell death. Serum may include factors affecting NF-κB signaling. To address this issue, we analyzed the effects of serum starvation on NF-κB binding to the target sequence by EMSA of nuclear extracts from several cell lines (ST1,

COS, and COS-A717) and mouse primary spleen cells. The binding of NF-κB to the target sequence was enhanced by serum starvation in all examined cells, except for the mutant COS-A717 cells, which exhibit defective NF-κB signaling (Fig. 1). These results indicate that serum starvation induces NF-κB activation, suggesting that serum contains unknown factor(s) inhibiting NF-κB activity.

Expression cloning of an NF-κB activating molecule using COS-A717 cells

Many transformed cell lines containing the COS cell line have constitutively activated NF-κB signaling. To identify NF-κB activators in the COS cells, a COS-A717 cell derivative containing the GFP gene under the control of the Sp1 site-deleted HIV-1 LTR was constructed, and the cells were designated as COS-A717-GS. The GFP is expressed by NF-κB activation in the COS-A717-GS cells, because the expression from the Sp1 site-deleted HIV-1 LTR is NF-κB dependent. The COS-A717-GS cells were transfected with a human spleen cDNA expression library, and GFP-expressing cells were selected (Fig. 2A). The sequence analysis of the cDNA expressed in the GFP-positive COS-A717-GS cells revealed that it perfectly matched the Gβ2. The expression level of the Gβ2 protein in COS-A717 cells was much lower than that in the parental COS cells (Fig. 2B).

To confirm that Gβ2 activates NF-κB activity, COS and COS-A717 cells were transfected with the Gβ2 expression plasmid, and the NF-κB promoter activity was measured using the 5 × κB-luciferase plasmid. Gβ2 activated the NF-κB promoter activity in both COS and COS-A717 cells, in a dose-dependent manner (Fig. 2C). Transfection of the COS-A717 mutant cells with the Gβ2 expression plasmid (0.5 μg) resored the NF-κB activity comparable to the parental COS cells (sevenfold). When 0.5 μg of the Gβ2 expression plasmid was transfected
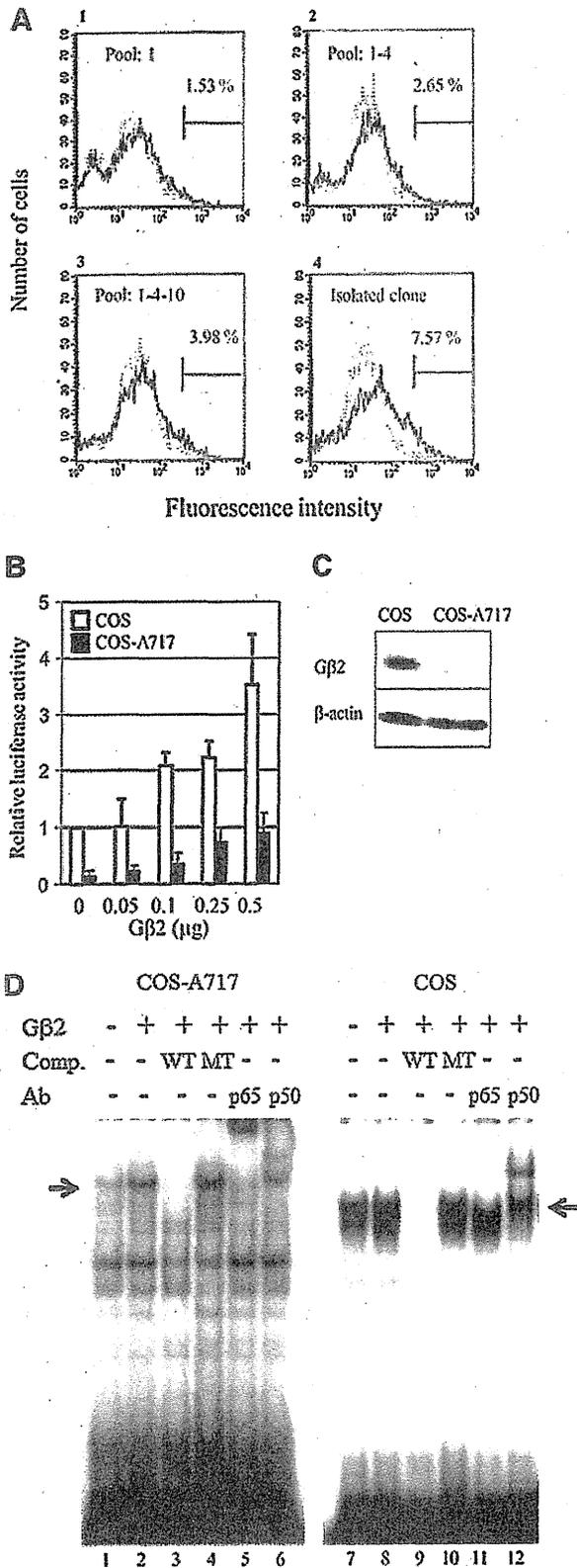
into the COS cells, the NF-κB activity was also increased by threefold. The DNA-binding activity of NF-κB was elevated by Gβ2 by about 1.7- and 6.2-fold in COS and COS-A717 cells, respectively (Fig. 3D). The complex formation was inhibited by a wild-type κB oligonucleotide competitor, but not by a mutant κB oligonucleotide. The complex was supershifted by both anti-p65 and -p50 antibodies, indicating that the complex consisted of p65 and p50 (Fig. 3D). The Gβ2 transfection activated the NF-κB signal more efficiently in the mutant COS-A717 cells than in the COS cells that originally express Gβ2. These results indicate that Gβ2 activates NF-κB signaling and the defect of Gβ2 expression is one of the determinants for the reduced NF-κB activity in the COS-A717 cells.

Gβ2 is required for NF-κB activation induced by serum starvation

Serum starvation activated NF-κB by 10-fold in COS cells, but had no effect in COS-A717 cells (Fig. 3A). Since the level of the Gβ2 protein is much lower in the COS-A717 cells than in the parental COS cells, we examined whether Gβ2 was involved in the NF-κB activation by serum starvation. Serum starvation elevated the NF-κB activity by fourfold in the Gβ2-transfected COS-A717 cells, indicating that Gβ2 is required for the serum starvation-induced NF-κB activation and that the Gβ2-activated signal is inhibited by the unknown factor(s) present in serum. However, because the level of NF-κB activity in the starved Gβ2-expressing COS-A717 cells was lower than that in the starved COS cells, the COS-A717 cells have additional defect(s) in the NF-κB signal activation.

FIG. 2. G protein β2 (Gβ2) activates NF-κB. (A) Identification of Gβ2 by expression cloning. (A-1) COS-A717-GS cells were transfected with plasmids obtained from a positive pool of 50 bacterial transformants (pool 1) following four rounds of FACS enrichment. (A-2) COS-A717-GS cells were transfected with plasmids from a positive pool (1-4), containing 20 bacteria colonies. (A-3) COS-A717-GS cells were transfected with plasmids from a positive pool (1-4-10), containing 10 bacterial colonies. (A-4) COS-A717 GS cells were transfected with a Gβ2-encoding clone. (B) Western blot analysis of Gβ2 in COS and COS-A717 cells. Proteins were analyzed by immunoblotting with an anti-Gβ2 Ab (top) and an anti-β-actin Ab (bottom). (C) Gβ2-mediated NF-κB activation in COS-A717 cells. COS and COS-A717 cells were transiently transfected with 0.25 μg of the 5× κB-luciferase reporter and the Gβ2 expression construct (0.05, 0.1, 0.25, and 0.5 μg), and then additional DNA (pcDNA3) was added to make the total amount of DNA 1 μg/well. At 48 h post-transfection, the cells were harvested and the luciferase activity was measured. The relative transfection efficiency in each sample was determined by the measurement of the Renilla luciferase activity. The relative luciferase activity in control COS cells (without Gβ2) was set to 1.0. Data shown are averages +SD from three independent experiments. (D) Gβ2 induced the NF-κB-binding ability in COS-A717 cells and COS cells. Nuclear proteins from untransfected (lanes 1, 7) or Gβ2 transfected (lanes 2–6, 8–12) COS-A717 cells or COS cells were isolated. The unlabeled consensus κB oligonucleotide (lanes 3, 9) or the mutant κB oligonucleotide (lanes 4, 10) was added as a competitor in a 20-fold molar excess to the binding reaction. Abs against p65 (lanes 5, 11) and p50 (lanes 6, 12) were added to the reaction for a supershift assay. The arrow indicates the NF-κB-containing complex.
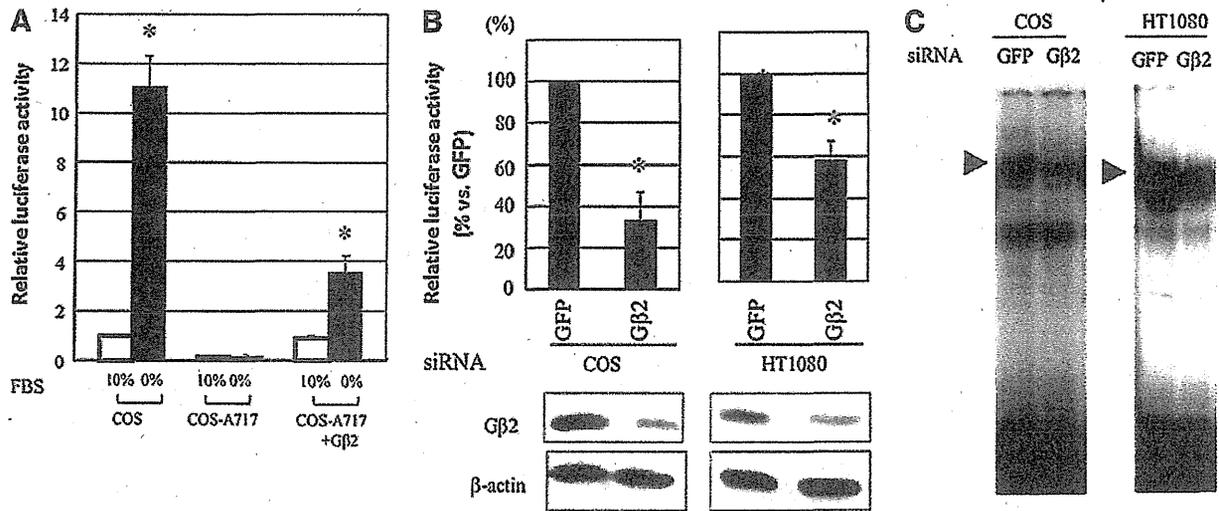
FIG. 3. Gβ2 is required for serum starvation-induced NF-κB activation and for constitutive NF-κB activation in transformed cells. (A) The serum-starving stress-induced NF-κB activation was analyzed by a luciferase assay. COS, COS-A717, and Gβ2-transfected COS-A717 cells were transiently transfected with the 5×κB-luciferase reporter. Six hours after transfection, the cells were washed with phosphate buffered saline (PBS) and incubated with (10%) or without FBS (0%) for 36 h for the luciferase assay. The luciferase activities in COS cells incubated with 10% FBS were set as 1.0. The activations were significant (*$p < 0.05$). (B) The Gβ2 siRNA reduced the NF-κB activity in COS and HT1080 cells. The NF-κB activity was determined by transfection with the 5×κB-luciferase reporter together with the GFP or Gβ2 siRNA, and shown as the % of that in cells transfected with the GFP siRNA. Western blot analyses of Gβ2 (top) and β-actin (bottom) in cells transfected with the GFP or Gβ2 siRNA#1 were performed. The inhibitions were significant (*$p < 0.05$). (C) Nuclear cell extracts were isolated from COS and HT1080 cells transfected with the GFP or Gβ2 siRNA. The arrowhead indicates the NF-κB-containing complex.

Gβ2 is involved in NF-κB in HT1080 human fibrosarcoma cell line

HT1080 cells also have a relatively high level of basal NF-κB activity. We examined whether Gβ2 contributes to the constitutive activation of NF-κB in HT1080 cells. Knockdown of Gβ2 expression by siRNA reduced the basal NF-κB activity not only in COS cells, but also in HT1080 cells (Fig. 3B). The siRNA against Gβ2 indeed reduced the Gβ2 protein level. Consistent with the κB promoter activity, the knockdown of Gβ2 inhibited the NF-κB-binding capability to the target sequence (Fig. 3C). These results indicate that Gβ2 is required for the constitutive activation of NF-κB in COS and HT1080 cells, suggesting that Gβ2-mediated signaling contributes to the constitutive NF-κB activation. The serum-deprived Gβ2-mediated NF-kB activation in COS cell was also confirmed using another siRNA (Supplementary Fig. S2).

Impact of IKKs, NEMO, and IκB in Gβ2-induced NF-κB activation

To determine whether the Gβ2 induced NF-κB activation requires IκB phosphorylation, a IκBα-SR with mutations at the inducible phosphorylation sites, S32G and S36A, was coexpressed with Gβ2 in COS-A717 cells. The IκB-SR abolished the Gβ2-induced NF-κB activation in a dose-dependent fashion (Fig. 4A). This result suggests that the phosphorylation of IκBα at S32 and S36 is necessary for the Gβ2-induced NF-κB activation. An important regulator of phosphorylation in the IκB pathway is the IKK complex, which comprises multiple kinases, including IKK1 (IKKα), IKK2 (IKKβ), and NEMO (IKKγ). We examined whether IKK1, IKK2, and/or

NEMO were involved in the Gβ2-induced NF-κB activation. Dominant negative forms of IKK1 (IKK1.DN), IKK2 (IKK2.DN), and NEMO (NEMO.DN) were each coexpressed in the Gβ2-transfected COS-A717 cells. As shown in Figure 4B–D, IKK1.DN, IKK2.DN, and NEMO.DN were each able to reduce the Gβ2-induced NF-κB activation in COS-A717 cells in a dose-dependent fashion, indicating that IKK1, IKK2, and NEMO are involved in the Gβ2-induced NF-κB activation. Especially, the IKK2.DN more efficiently suppressed the Gβ2-mediated NF-κB activation than IKK1.DN and NEMO.DN. This result suggests that IKK2 plays an important role in the Gβ2-induced NF-κB activation, like the bradykinin-induced NF-κB activation through Gαq and Gβγ (Xie et al., 2000). To confirm the expressions of the dominant negative and endogenous IKK1, IKK2, NEMO, IκBα, and Akt, COS cells were transfected with the mutant expressing plasmids, and the cell lysates were subjected to Western blotting analysis using their specific antibodies. The descriptions of the dominant negative mutants and their specific antibodies used here are summarized in the Tables 1 and 2, respectively. As shown in Figure 5A, we have confirmed that the anti-IKK1, NEMO, and Akt1 antibodies were able to detect the simian endogenous proteins as well as their human and mouse dominant negative mutants. On the other hand, the antibodies against IKK2 and IκBα reacted to their human dominant negative mutants and the human endogenous proteins in HEK293T cells, but not to the simian endogenous proteins. Considering the different affinities of the antibodies between endogenous simian proteins and their human or mouse counterparts, we could not assess precisely the relative amounts of dominant negative mutants to endogenous proteins in COS cells. However, each
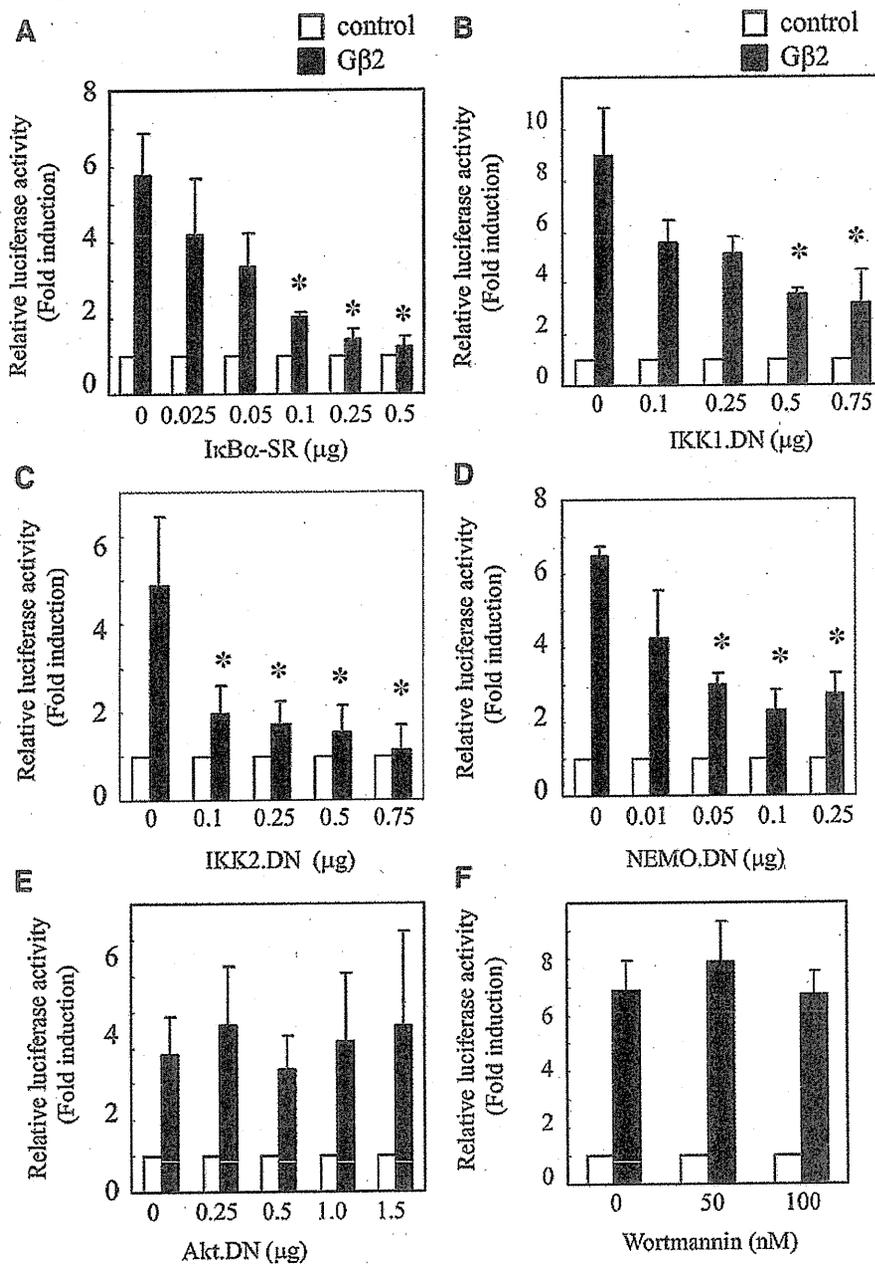
FIG. 4. Involvement of IκB, IκB kinase 1 (IKK1), IKK2, and NEMO in Gβ2-induced NF-κB activation. COS-A717 cells were transfected with 0.25 μg of the 5×κB-luciferase and the mutant expression plasmid of IκBα super-repressor (IκBα-SR) **(A)**, IKK1 (IKK1.DN) **(B)**, IKK2 (IKK2.DN) **(C)**, NEMO (NE-MO.DN) **(D)**, or Akt (Akt.DN) **(E)**, together with the Gβ2 construct (0.25 μg) or pcDNA3.1. The pcDNA3.1 plasmid was added to make the total amount of DNA 1 μg/well. COS-A717 cells were cotransfected with 0.5 μg of the 5×κB-luciferase reporter without (control) or with 0.5 μg of the Gβ2 construct. Wortmannin was added 1 h before transfection **(F)**. The relative luciferase activity in the control cells without Gβ2 was set as 1.0. Data shown are averages and SD from three independent experiments. The inhibitions were significant (*$p < 0.05$).

TABLE 1. CHARACTERISTICS OF THE DOMINANT NEGATIVE MUTANTS

Molecule	Species	Structure	MW (kDa)	Tag
IκBα	Human	S32G, S36A mutant	40	—
IKK1	Human	N145D mutant	85	VSV
IKK2	Human	N145D mutant	87	VSV
NEMO	Mouse	97–412 C-terminal fragment	37	VSV
Akt	Mouse	K179A, T308A, S473A mutant	69	—

MW, molecular weight.

TABLE 2. CHARACTERISTICS OF THE ANTIBODIES

Antigen	Cross-reactivity
Human IκBα C-terminal peptide	Human/mouse/rat
Human IKK1 full-length	Human/mouse/rat
Human IKK2 C-terminal peptide	Human/canine
Human NEMO full-length	Human/mouse/rat
Human Akt1 345–480 peptide	Human/mouse/rat
Human phospho-S32 and S36-containing peptide	Human/mouse