



FIG. 4. Insertion of the RSV F cleavage site II in SeV F leads to trypsin-independent syncytium formation. BSR-T7/5 cells growing in microchamber wells were cotransfected as previously described with 0.25 μ g pTM1-F_RKR or pTM1-Fc mutants and 0.25 μ g pTM1-HN plasmid (+HN) or 0.25 μ g empty pTM1 vector (–HN). The transfection mixture was removed 7 h posttransfection, and cells were incubated in serum-free medium with (+) or without (–) trypsin. Cells were fixed 32 h posttransfection and subjected to immunostaining as described in Materials and Methods.

Fc_{103–110} mutant forms syncytia in the absence of HN. However, the nature and/or length of the intervening residues between the two cleavage site does appear to influence fusion to a greater extent in the absence of HN, since Fc_{103–117} formed large syncytia whereas Fc Δ _{103–117} formed only small syncytia without HN.

In the absence of HN coexpression, three mutants containing both F_{RSV} cleavage sites (Fc_{103–110}, Fc_{103–117}, and Fc_{103–130}) produced fusion activity in the luciferase reporter gene assay in the presence of trypsin (Fig. 6B). As observed in the syncytium formation assay, Fc did not produce significant fusion in the absence of HN. Fc Δ _{103–117} also failed to produce HN-independent fusion, reflecting the small syncytia produced by this mutant in the absence of an attachment protein, in spite of the fact that it contains both F_{RSV} cleavage sites.

Enhanced membrane fusion in the absence of HN by the double cleavage site mutants paralleled the increase in F0-to-F1 cleavage by trypsin, compared with single site mutants (Fig. 3B). However, no precise quantitative correlation between F0 processing and membrane fusion activity in the absence of HN could be found. For instance, while Fc Δ _{103–117} was highly susceptible to trypsin cleavage, this mutant was poorly active in both membrane fusion assays (Fig. 6). Furthermore, although Fc_{103–130} was cleaved by trypsin more efficiently than Fc_{103–110} or Fc_{103–117} (Fig. 3B), it did not produce larger syncytia or induce greater luciferase activity in the absence of HN (Fig. 6).

The Fc_{103–117} mutant produced the greatest fusion activity in the absence of the HN protein and was subjected to a dose-response fusion assay in the presence of trypsin and compared with Fc (Table 1). Increasing quantities of DNA were used to transfect BSR cells, and the corresponding increase in cell surface expression was determined by flow cytometry. An average fusion index was subsequently calculated from the fusion and expression data. While increasing expression of Fc did not enhance fusion, a dose response of fusion was seen for Fc_{103–117}, confirming the ability of this double cleavage site mutant to fuse cells in the absence of HN. The fusion index

estimated for Fc_{103–117} was 0.72, compared to 0.08 for the Fc mutant and 1.0 for wild-type F (F_{SeV} coexpressed with HN in the presence of trypsin). However, higher expression levels of Fc_{103–117} were required in the luciferase reporter gene assay, compared to the syncytium formation assay, in order to observe a level of HN-independent cell-cell fusion comparable to the wild type. This may reflect differences in the requirements of the two assays. For instance, the presence of F protein in both target and donor cells in the syncytium formation assay may facilitate cell-cell fusion with respect to the reporter gene assay, in which F protein is present only in the donor BSR cells. Furthermore, the reporter gene assay involves the interaction between two distinct populations of cells and may depend to a greater extent on the attachment ability of the mutated F proteins.

Coexpression of the attachment G protein fails to restore the reduced fusogenic capacity of RSV F cleavage site mutants. It has previously been shown that cleavage at both sites I and II is a requirement for fusion of the F_{RSV} protein, expressed in transfected cells in the absence of the attachment G protein. Mutations of F_{RSV} cleavage site I abrogated syncytium formation in the absence of trypsin, whereas deletion of F_{RSV} cleavage site II prevented syncytium formation, even in the presence of trypsin (12, 45). In light of our findings that insertion of F_{RSV} cleavage site I in F_{SeV} led to syncytium formation by F_{SeV} in the absence of HN, we aimed to determine whether coexpression of RSV G protein with F_{RSV} cleavage site mutants would restore cell-cell fusion.

A series of F_{RSV} mutants (Fig. 7A), which have been previously described (12, 28, 29), were subcloned in the pTM1 plasmid. These mutations included changes in cleavage site I (RARR) to produce the F_{R108/109N} mutant (RANN) or deletion of four basic residues in cleavage site II (KKRKRR), resulting in the F Δ _{131–134} mutant (12). Further deletions in F_{R108/109N} resulted in the RSV F Δ _{108–120} and F Δ _{108–132} mutants (28, 29). All mutant proteins were expressed at the cell surface at a level comparable to F_{RSV} (Fig. 7B), with the exception of the cleavage site II mutant F Δ _{131–134}, which