

ABSTRACT

The formin family of proteins contain unique Formin Homology (FH) domains required for their association with actin-binding profilin (FH1) and nascent or mature actin filaments (FH2). These domains are essential for maintenance of the cytoskeletal elements as well as the nucleation, growth, and depolymerization of actin. INF1 is different from other formins as it contains both formin homology domains FH1 and FH2 at its N-terminus as well as a microtubule binding domain at its C-terminus. The presence of these motifs suggests a strong role in regulation and interaction between both the actin and microtubules of the cytoskeleton. Overexpressed INF1 protein from transfected cell cultures is observed to have a larger molecular weight than the endogenous protein. This difference in molecular weight is suggested to have arisen as a result of the post translational modifications such as proteolytic degradation of a proposed INF1 precursor. In order to test this hypothesis in future experiments, antibodies were raised and tested for their efficacy in recognizing specific residues on the endogenous, overexpressed, mouse and human INF1.

INTRODUCTION AND OBJECTIVES

- INF1 is 1143 residues and migrates on SDS-Page with a relative mobility of 160 kDa. Surprisingly, the endogenous protein migrates at 130kDa. This difference in molecular weight is hypothesized to result from post-translational processing of the endogenous protein.
- Immunofluorescence and western analysis assays were used to assay the ability of two new antisera, 968C and FHDC1, to recognize over-expressed and endogenous INF1 protein.
- Immunofluorescent microscopy is conducted to reveal the subcellular localization of the endogenous INF1 with respect to fluorescent protein tags and antibodies of interest.

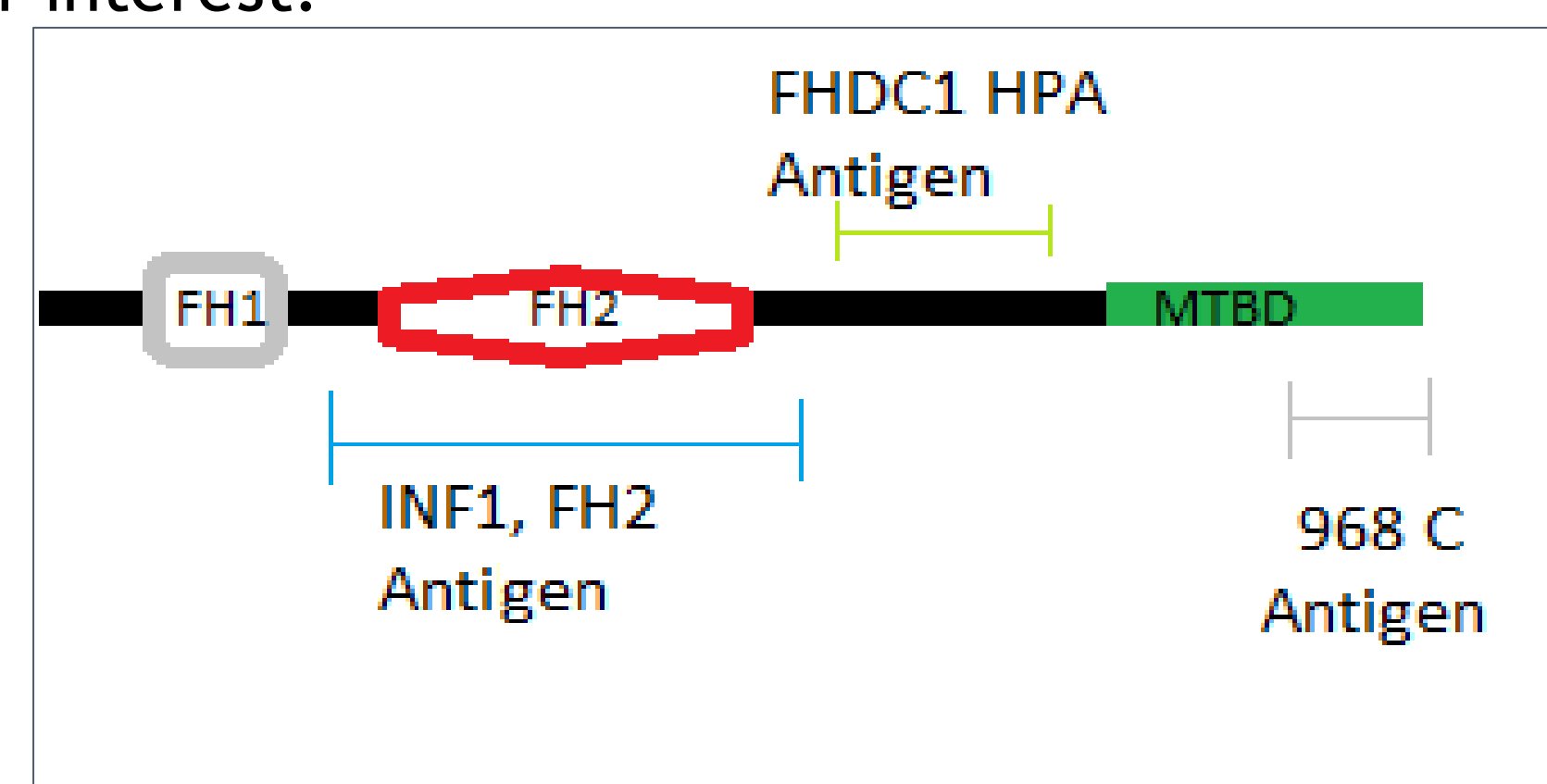


Figure 1: Diagram of the INF1 formin protein and its functional formin homology and microtubule binding domains. In light blue, the approximate epitope of the α INF1 FH2 antibody. In light green, the epitope of the α FHDC1 HPA antibody, and in grey, the 968 C epitope is shown.

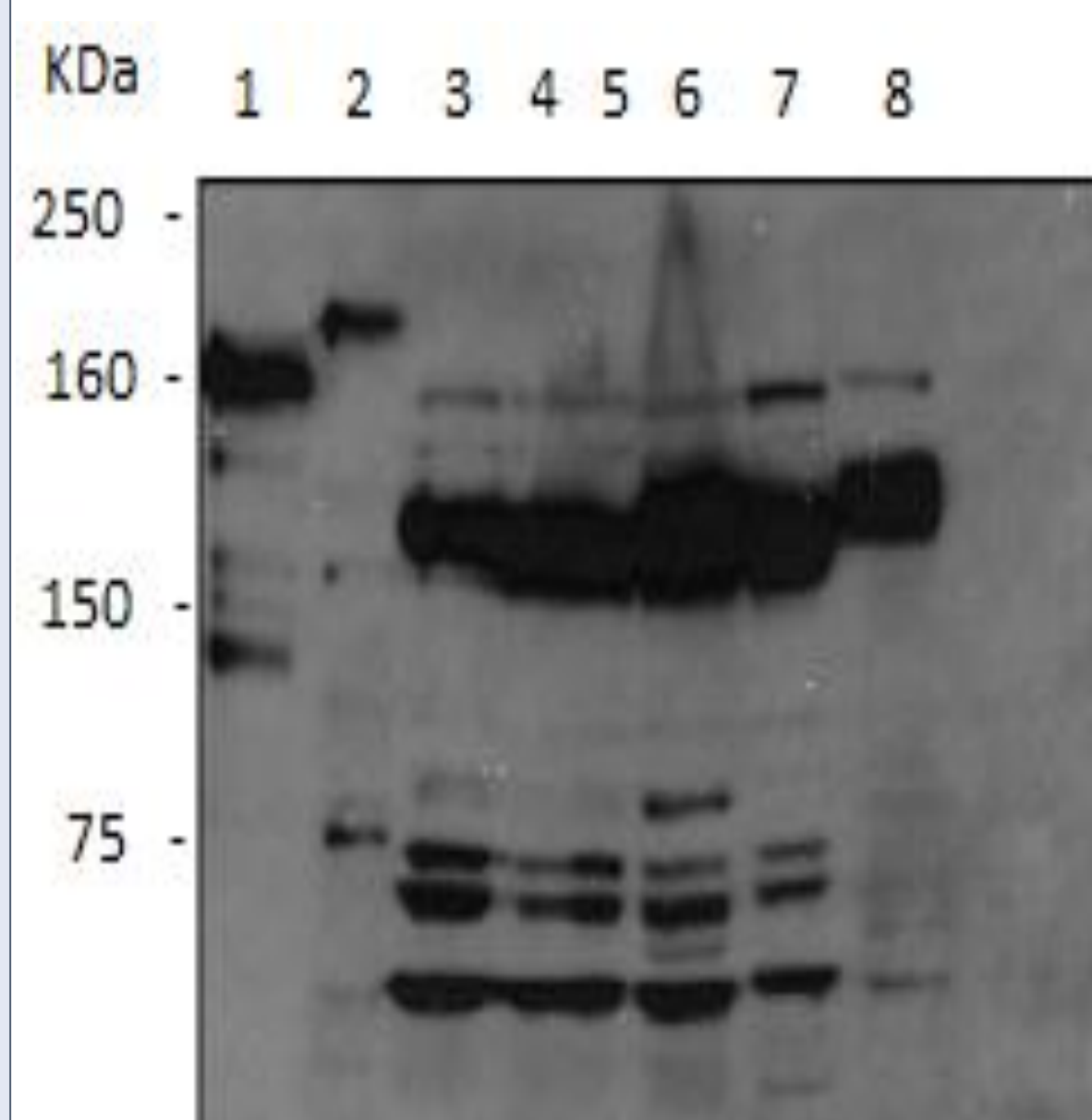


Figure 2: Immunoblot Assay conducted to compare overexpressed INF1 protein to the endogenous species. (1) Assay conducted on myc-INF1FL overexpressed INF1 protein running at 160 kD (2) GFP tagged GFP-mINF1 with a slightly higher molecular weight due to the addition of a tag (3-5) endogenous neuronal cell lines (NZA and F11 (6) fibroblast, (7) cardiomyoblast, and (8) HeLa cell lines

RESULTS

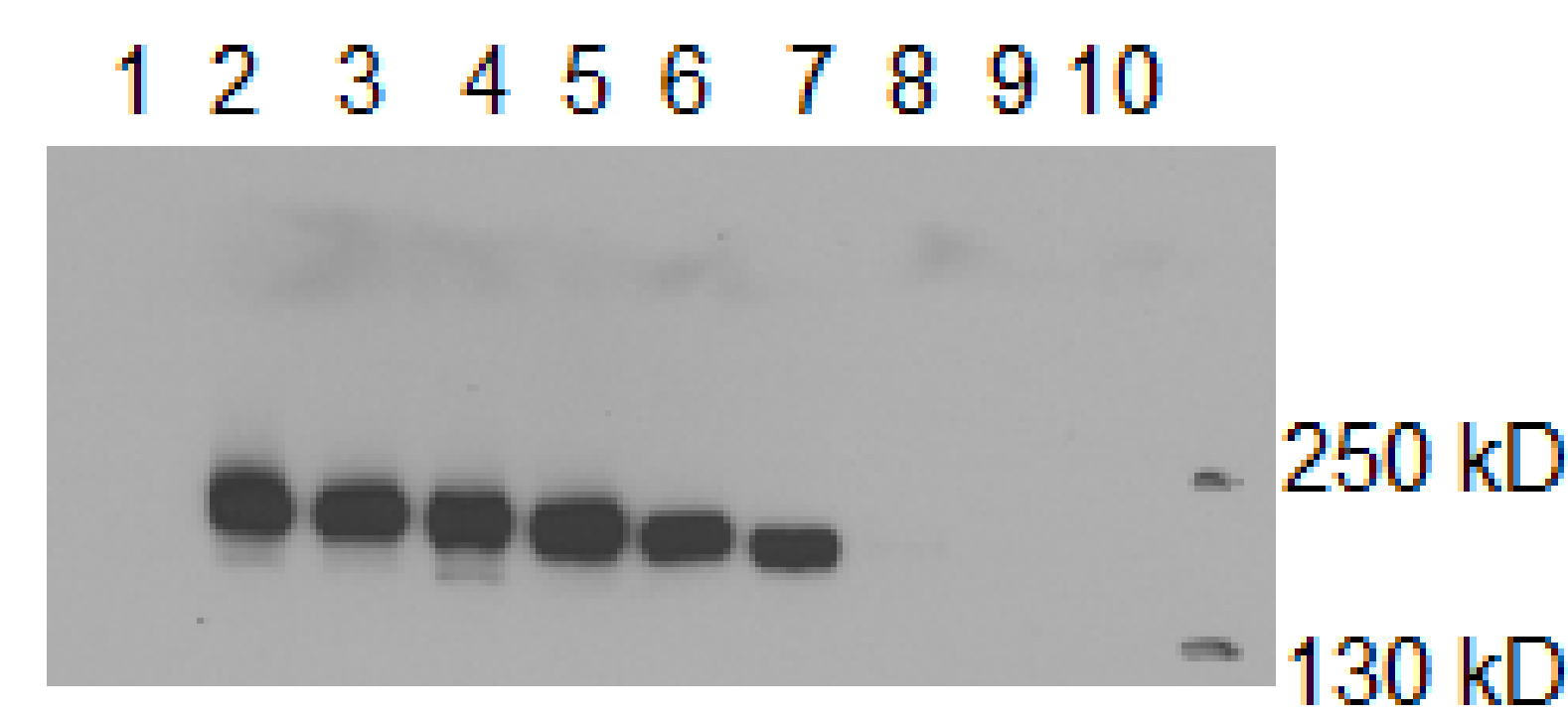


Figure 3: Immunoblot Analysis of INF1 derivatives using the C-terminal α 968C antibody. (1) Negative control extract not containing INF1 Protein. (2-10) Extracts containing RFP-tagged INF1 derivatives. Constructs used are 1-1143, 1-1123, 1-1086, 1-1055, 1-1033, 1-1004, 1-978, 1-967, and 1-958 in lanes 2-10 respectively. INF1 derivatives containing the 968C epitope were detected using Rabbit anti-INF1 primary antiserum and a donkey anti-rabbit secondary antibody coupled to horse radish peroxidase (HRP). The deletion of aa residues 978 to 1004 results in loss of detection by 968C, (compare lane 8 to lane 7).

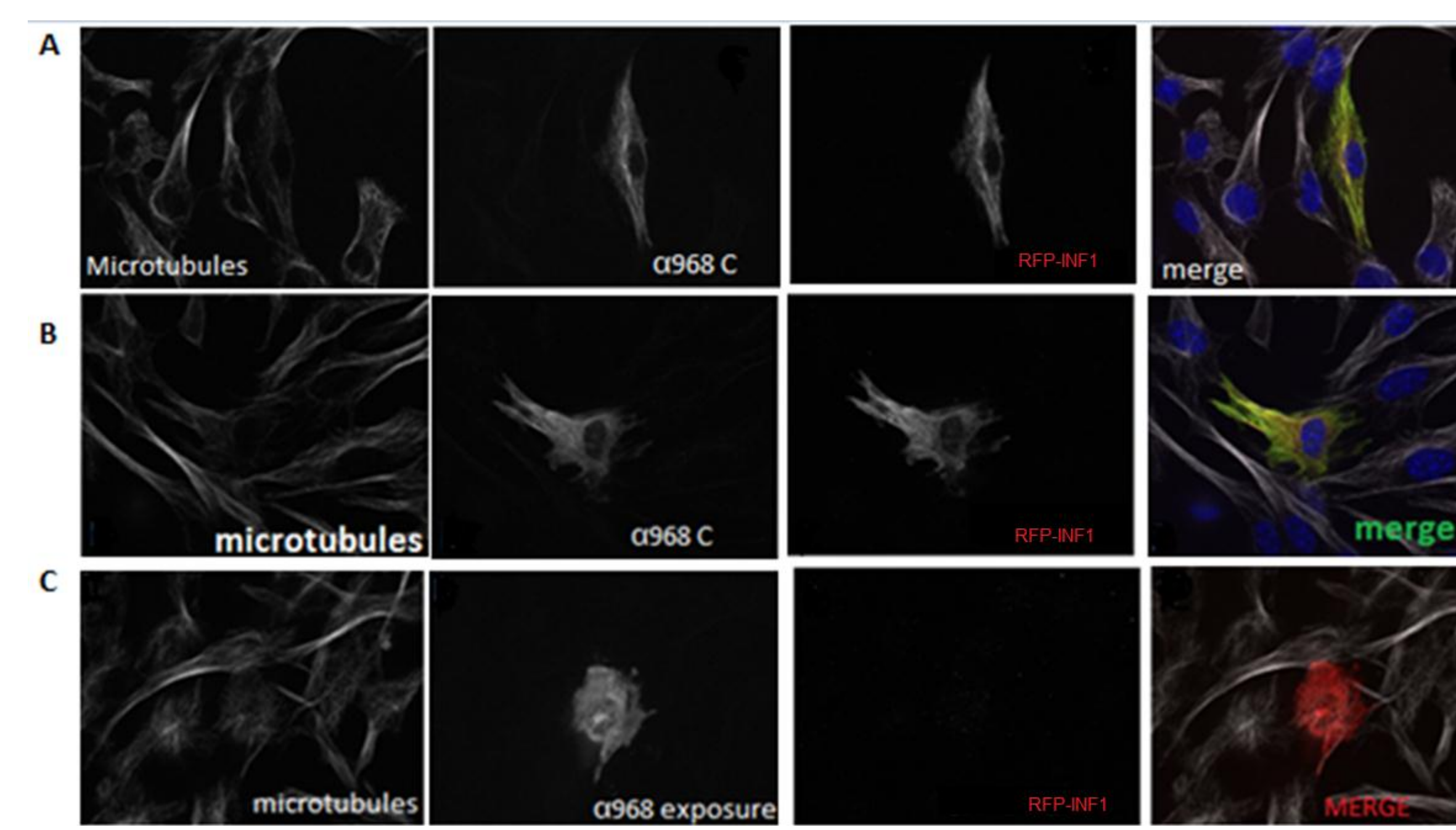


Figure 4 : Full length, 1-1004, and 1-978 over expressed INF1 protein constructs exposed to α 968C antibody. (A) Full length overexpressed RFP-human INF1 and (B) RFP-human INF1 1-1004 are efficiently recognized by the 968C antisera while RFP-human INF1 1-978 is not. INF1 protein was detected by virtue of its RFP tag as a positive control for levels of expression.

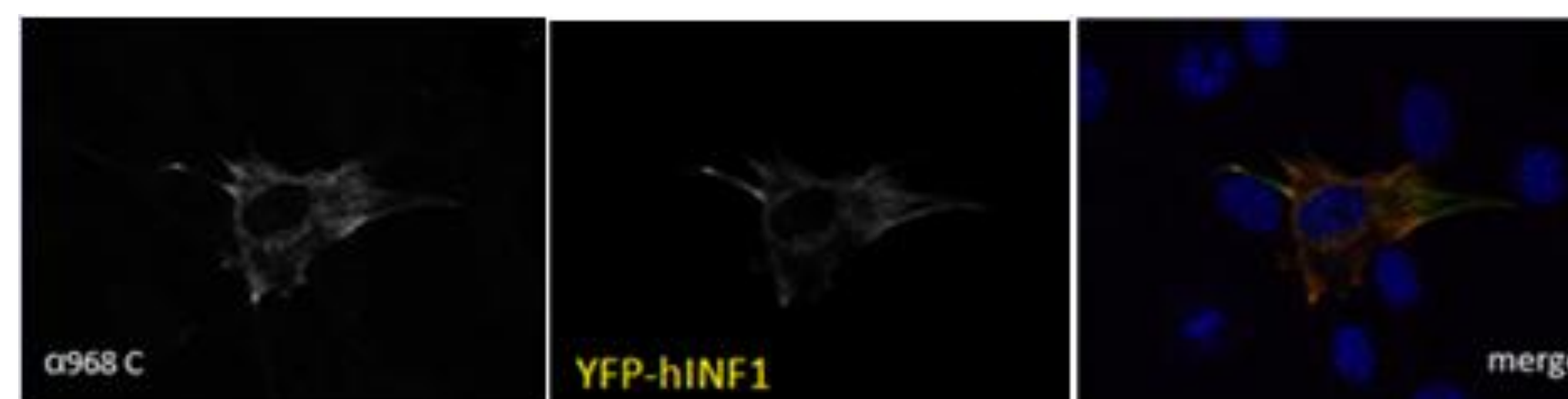


Figure 5: YFP tagged human INF1 protein is recognized by the α 968 C antibody.

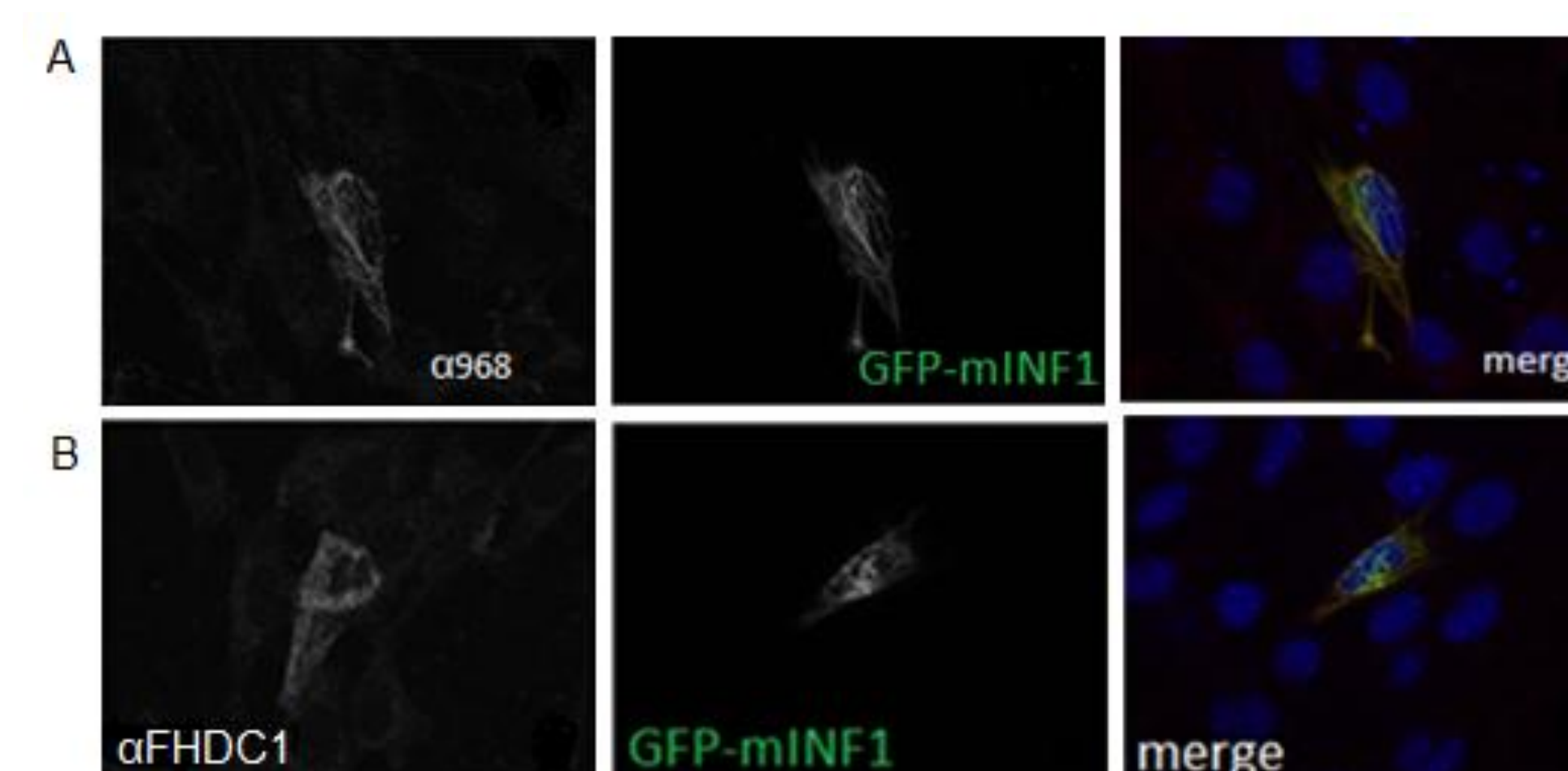


Figure 6: Mouse INF1 detected with α 968 C and α FHDC1 antisera. GFP-tagged mouse INF1 protein was expressed in mouse fibroblast cells and visualized by virtue of its GFP tag. The 968C (A) or FHDC1 (B) antisera were tested for their ability to detect the over-expressed mouse homologue of INF1.

ANALYSIS OF RESULTS

- The 1-1004 INF1 derivative was efficiently recognized by the 968C antiserum while the 1-978 derivative was not. This suggests that the primary epitope for this lies between residues 978-1004 of the human INF1 protein.
- Immunofluorescence assays show that the C-terminal antibody binds very well to both mouse and human INF1
- Immunofluorescence assays suggest that the α FHDC1 antisera is able to recognise human INF1 protein, but with a much lower affinity than 968C. Additionally the affinity of the FHDC1 antiserum for mouse INF1 protein is too low to make it of practical use for experiments with the endogenous protein expressed in cells of mouse origin.

METHODOLOGY

- NIH 3T3 Mouse Fibroblast cells Seeded for transfection
- Transfection of cells via uptake of pEFINF1 and pHINF1eyf vectors encoding genes for various INF1 constructs
- In order to map the antigen of the C-terminal antibody, the following constructs of the full length (1143 aa) INF1 protein were created: 1-1143, 1-1123, 1-1086, 1-1055, 1-1033, 1-1004, 1-978, 1-967, and 1-958.
- Purification of INF1 Protein from transfected cells
- Western Analysis of purified protein using C-terminal antibody to map antigenic region of INF1
- Cells were fixed for immunofluorescence assay using Paraformaldehyde, and Methanol to observe differences in antibody binding based on method of fixation
- Immunofluorescence assay conducted on slides mounted with primary antibody of interest, followed by secondary fluorochrome-conjugated antibodies
- Cells stained using BAPI and mounted for examination on the Zeiss AxioImager microscope with attached Apotome

CONCLUSION

It was determined that the C terminal antigen resides between 978 and 1004 at approximately residue 968 (figure 3). This region is highly conserved between mouse and human and thus observed clarity is fairly similar. Slight differences due to mismatching residues can be accounted for by using a slightly higher concentration of antibody. The α FHDC1 proved to be a weak antibody for mouse endogenous INF1 as it was specifically created to the less conserved section of hINF1. Thus α FHDC1 will not be used in further studies of INF1 as it must be used in very high concentrations to yield decent results (figure 6). Western analysis of the endogenous protein determines the molecular weight of INF1 to be 130 kD. However, the over-expressed INF1 in transfected cells rejects this prediction. This finding is consistent with the hypothesis for the post translational proteolytic modification of endogenous INF1.

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- Any inquiries can be directed to Shabnam Saidzada (ssaid067@uottawa.ca)

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