VEGF-C utilizes both of its potential N-glycosylation sites

2 µg of purified mature VEGF-C was subjected to enzymatic deglycosylation under denaturating conditons for 24h by PNGase F (New England Biolabs, Ipswich, MA) according to the standard procedures of the manufacturer. Similarly, 1 µg of purified mature VEGF-C was digested by Endoglycosydase H. Digestion products were resolved on a reducing, 4-12% gradient SDS-PAGE gel and visualized by silver staining. Undigested protein migrated as two bands of approximately 19 and 21 kDa. After digestion with both enzymes the apparent molecular mass was reduced to less than 16 kDa, but significant amounts of non-digested VEGF-C remained, even when enzyme and digestion times were increased 5-fold. To show that the remaining size heterogeneity is due to cleavage-resistant glycosylation, we created three different glycosylation mutants, in which the first, the second or both asparagine residues of the two potential N-linked glycosylation sites were mutated into glutamine residues. VEGF-C and its glycosylation-deficient mutants were immunoprecipitated from conditoned medium after transient transfection and metabolic labeling of 293T cells with VEGFR-2/IgGFc or VEGFR-3/IgGFc fusion protein, resolved on a reducing 16% SDS-PAGE gel and visualized using a phosphoimager plate. Expectedly, the completely unglycosylated VEGF-C (N12Q) was not secreted into the medium. Wild type VEGF-C migrated as double band of about 21/19 kDa. Both single glycosylation-deficient mutants migrated at about 19 kDa showing that both potential N-glycosylation sites are actually utilized in VEGF-C.