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Determination of differential phosphorylation and biological responses to EGF and nucleotides

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Protein phosphorylation is a dynamic post-translational modification and features, such as site occupancy and dynamics of modification at specific residues of proteins, are known to affect protein stability and function. To investigate the phosphoproteome profile of Human Corneal Limbal Epithelial cells following injury/nucleotide or growth factor stimulation, we have designed and carried out mass spectrometry-based quantitative phosphorylation analyses. Particular attention was paid to maximizing recovery of phosphoproteins/peptides and minimizing artifactual loss of phosphate during the LC/MS analysis. Cells were injured with a scratch wound and their migration in response to individual nucleotides was monitored, as was the expression of P2Y receptor mRNA ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$). Over 16 h, UTP and UDP stimulation resulted in 100% wound closure; ADP and ATP stimulation resulted in 30% and 70% wound closure, respectively. The only receptor that changed over time was $P2Y_2$, which demonstrated a significant increase as cells migrated. To test the role of $P2Y_2$ in the nucleotide-induced phosphorylation of the observed sites, we employed stable isotope labeling of amino acids in cell culture and transfection of siRNA directed to $P2Y_2$ and $P2Y_4$ receptors. Mass spectrometry-based analysis of the phosphorylation profiles showed a change in the phosphorylation of EGFR tyrosine residues in the $P2Y_2$ knockdown cells and Western blots using site-specific antibodies on cultures transfected with siRNA to $P2Y_2$ and $P2Y_4$ receptors showed a consistent decrease in phosphorylation of EGFR-Y845, EGFR-Y1068, and EGFR-Y1086 residues. In addition to the EGFR sites, phosphosites on many other proteins, notably protein kinase C, MAPK-1, -12 and -13, were differentially modified. Overall, our findings suggest that ligand-specific distinct phosphorylation of signaling proteins may dictate specific downstream signaling events.