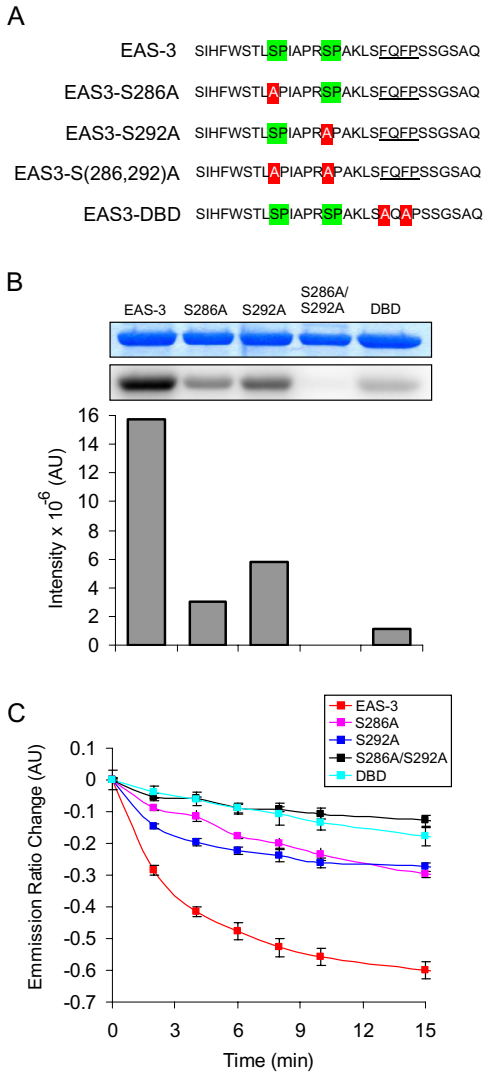

Figure 3

Mutation of key residues diminishes EAS-3 phosphorylation by pERK2. (A) Primary sequences of EAS-3 and EAS-3 mutant linker peptides are shown with consensus phosphorylation sites (highlighted in green) and ERK binding sites (underlined). Residue mutations to alanine are highlighted in red. (B) Analysis of mutant EAS-3 proteins by γ-[^{32}P]ATP phosphorylation shows complete loss of pERK2 phosphorylation of the S(286,292)A mutant as compared to wild-type EAS-3. Decreased ^{32}P-phosphorylation of site-specific mutants also indicates the involvement of both Ser286 and Ser292 for pERK2 activity. Requirement of the ERK binding domain for efficient phosphorylation is evident from decreased phosphorylation of the EAS3-DBD mutant. Band intensities were quantitated by densitometry and are shown graphically. Equal protein loading is shown by Coomassie staining of EAS-3 and EAS-3 mutants and phosphorylation assay was terminated after 15 minutes at 30°C. (C) Fluorimetry data for EAS-3 and EAS-3 mutants reveals that the FRET efficiency change is reduced with various mutations, consistent with the phosphorylation assay in B.

upon incubation with pERK2. We refined EAS-3 by replacing the bulky N-terminal GST-purification tag (Glutathione-S-Transferase) with a Histidine-10 tag on the C-terminus. This reduced the possibility that the large purification tag would interfere with FRET efficiency changes. In addition, we mutated alanine 207 and alanine 487 to lysine. As previously reported, this prevents EGFP dimerization [15]. This latter modification reduced co-purification of truncation products with full-length EAS (data not shown).

To further characterize EAS-3, we generated different constructs targeting critical residues, and analyzed their ability to serve as substrates for pERK2. Mutants EAS3-S286A, EAS3-S292A, and the double mutant EAS3-S(286,292)A eliminated consensus phosphorylation sites (Figure 2A). The dead binding domain mutant (EAS3-DBD) eliminated the DEF domain by replacing the two key phenylalanines with alanines, creating an AXAP motif (Figure 3A). As Figure 3B demonstrates, all five mutants have decreased phosphorylation compared to the wild type sensor. As expected, the EAS3-S(286,292)A mutant had the lowest level of phosphate incorporation due to absence of both serine-proline consensus phosphorylation sites required by MAP Kinases. A decrease in phosphorylation of EAS-DBD iss also consistent with the requirement of MAPKs to bind targets for efficient phosphorylation (reviewed in [16]). Differences between wild-type EAS-3 and mutants are also reflected in change of FRET efficiency when treated with pERK2 in fluorimeter experiments (Figure 3C). These relative changes in FRET efficiency are consistent with the radioassay data.

EAS-3 is not phosphorylated by pSAPK or pp38

Distinguishing the activation of different MAP kinases within the cell is essential since each MAPK pathway is activated by multiple mitogens and external environmental factors to varying degrees (reviewed in [17]). Furthermore, there is extensive cross-talk between the different MAP kinase pathways. Detection methods must effectively isolate the signal of the target kinase from other family members to elucidate the contributions of these different pathways to a given cellular process.

The target peptide linkers were designed to impart specificity for ERK. Elk1 and Ets1 were chosen because of their seemingly specific interaction with ERK relative to p38 or SAPK/JNK (reviewed in [18]). To determine whether EAS-3 acted as a specific substrate for ERK we performed *in vitro* phosphorylation assays to quantify the ability of pERK2, pp38, and pSAPK/JNK to phosphorylate EAS-3 (Figure 4). As shown in Figure 4A, EAS-3 is an approximately 2000-fold more specific target for pERK2 than pp38, and 50-fold more specific for pERK2 than pSAPK/JNK. Activities of pERK2, pp38 and pSAPK/JNK were