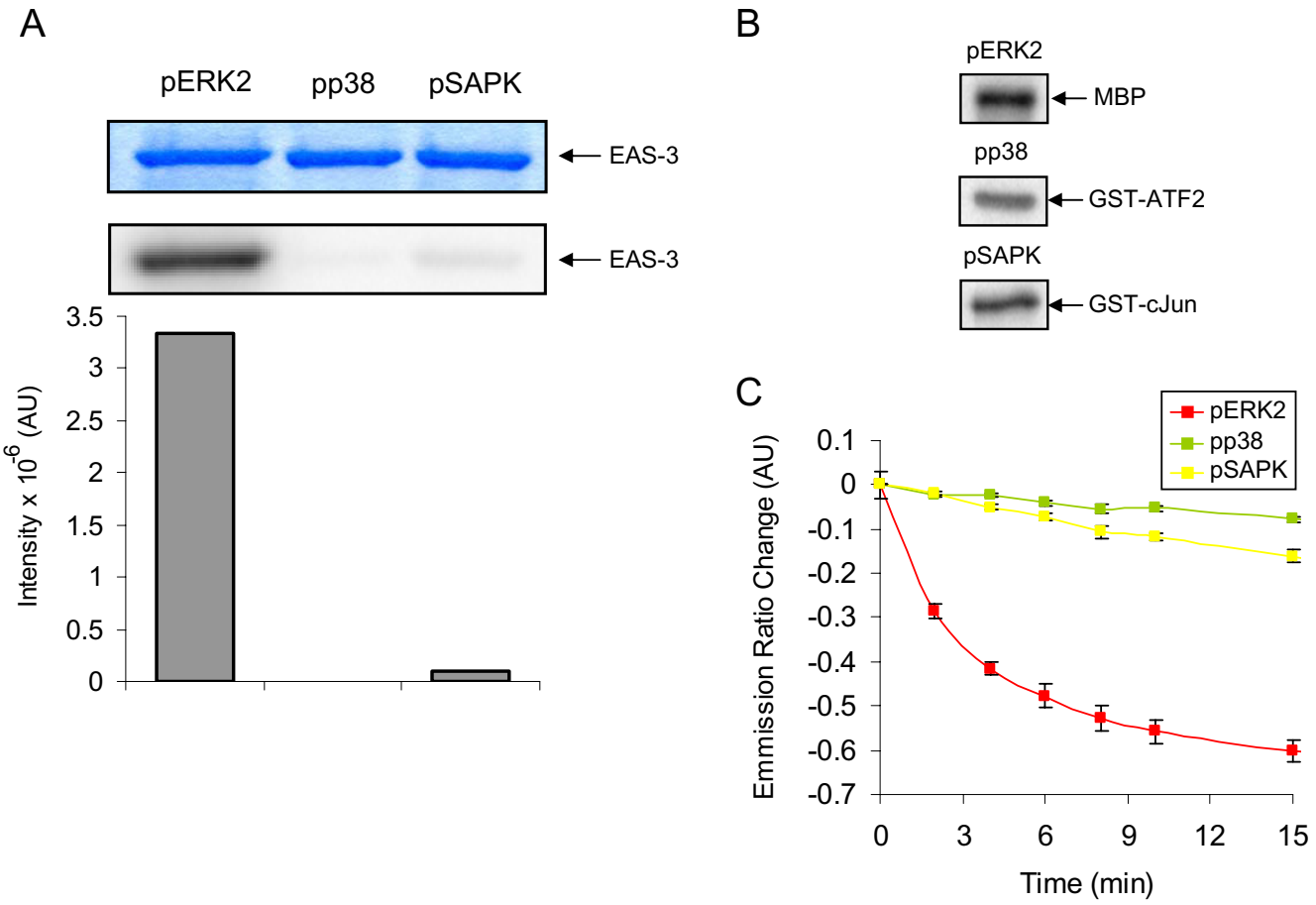

Figure 4

Determination of ERK2 specificity for EAS-3. (A) Phosphorylation of EAS-3 is efficient with pERK2, but not pp38 or pSAPK. pp38 and pSAPK incorporates little or no ^{32}P into EAS-3 at the same relative specific activity as pERK2. Band intensities were quantitated by densitometry and are shown graphically. Equal protein loading of EAS-3 is shown by Coomassie staining. (B) Specific targets for pERK2 (MBP), pp38 (GST-ATF2), and pSAPK (GST-cJun) are efficiently phosphorylated in the presence of γ-[^{32}P]ATP. The amounts of active kinase used in A were based on the relative incorporation of ^{32}P into each cognate substrate by the respective kinase. (C) Fluorimetry of EAS-3 with MAPKs shows that the FRET efficiency change is significantly diminished in the presence of pp38 or pSAPK as compared to pERK2. This is consistent with the phosphorylation assay in A.

confirmed by using known substrates specific for each kinase (Figure 4B). MBP was used in conjuction with pERK2, GST-ATF2 with pp38, and GST-c-Jun with pSAPK/JNK. Specificity of these kinases for EAS-3 was also confirmed by FRET in a fluorimeter (Figure 4C). It is clear that the change in FRET was dramatic when EAS-3 was incubated with pERK2, but showed little or no response when incubated with pp38 or pSAPK/JNK. This is consistent with the results found in 4A. Given the similarity of these kinases, this also suggests that EAS-3 would be specifically acted upon by pERK2 even in the presence of other activated intracellular kinases.

The demonstrated specificity of pERK2 for EAS-3 phosphorylation suggests that this sensor is a candidate for *in vivo* studies of ERK signaling. However, our preliminary experiments in NIH-3T3 cells indicated that EAS-3 was susceptible to intracellular phosphatase activity (data not shown). We surmise that this is due to the absence of a protective phospho-specific binding domain within the EAS-3 construct. Such binding domains have been crucial in the development of other FRET-based signaling sensors of kinase activity [2-4,6,7]. These binding domains, however, are taken from naturally occurring domains that are specific for each target sequence. Unfortunately, no known phospho-specific binding domain exists for Elk1