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ScienceDirect
Molecular Immunology 44 (2007) 3085–3091
Molecular Immunology
www.elsevier.com/locate/molimm

Epi-allelic Erk1 and Erk2 knockdown series for quantitative analysis of T cell Erk regulation and IL-2 production ☆

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Received 6 November 2006; received in revised form 21 January 2007; accepted 7 February 2007

Abstract

Erk activation is often used as a downstream pathway indicator of TCR signaling, generally in terms of both Erk1 and Erk2 isoforms measured together. In order to investigate potential distinctions between Erk1 and Erk2 regulation and effects downstream of TCR ligation, we generated a series of stable and independent Erk1 and Erk2 shRNA knockdown lines in the 1B6 T cell hybridoma. We observed no compensatory effect by opposite isoform upregulation, and found similar fractions of total phosphorylated Erk1/2 across this epi-allelic series in response to both anti-CD3 and peptide-MHC stimulation of TCR. Moreover, a previous prediction of an isoform-independent linear relationship between Erk activation and IL-2 production was confirmed. The effect of the shRNA-mediated knockdowns in reducing IL-2 production was observed to be stronger than that arising from pharmacological MEK inhibition at comparable degrees of ERK1/2 phosphorylation levels.

Keywords: Signal transduction; Cell activation; T cells; shRNA

1. Introduction

T cell receptor (TCR) ligation and cross-linking is known to result in the activation of Erk1/2 (MAPK1/2). Erk activation has been shown to be necessary for Jun and Fos complex to form the AP-1 transcription factor. AP-1 along with other transcription factors, binds to the IL-2 promoter resulting in IL-2 production (Whitehurst and Geppert, 1996; Li et al., 2004; Dumont et al., 1998).

Erk dynamics have been implicated in significantly helping the regulation of T cell phenotypic behaviors. Sustained versus transient Erk dynamics have been observed in naïve T lymphocytes, corresponding to positive and negative selection, respectively (Werlen et al., 2000). Furthermore, the duration of Erk signaling appears to influence CD4+CD8+ lineage commitment (Nishida et al., 2004). Multiple mechanisms have been postulated to generate these differences in Erk dynamics. Recently, a positive feedback loop in which Erk phosphorylates Lck in a regulatory manner to prevent SHP-1 deactivation of Lck was shown to sustain TCR signaling (Stefanova et al., 2003). A mathematical modeling effort has demonstrated how the balance between this novel Erk positive feedback and negative feedback via phosphatase-mediated deactivation of molecules proximal to the TCR allows for sensitivity in ligand discrimination (Altan-Bonnet and Germain, 2005). A downstream positive feedback loop between Erk-mediated immediate early gene expression (Fischer et al., 2005) and Erk has also been described (Murphy et al., 2002). The dynamic integration of multiple feedbacks both upstream and downstream may yield a strong dependence of T cell responses to Erk activity. To this point, we have recently experimentally validated a computational model prediction of a linear relation-

☆ Funding for this work was provided by grants from the National Institutes of Health (NIGMS Cell Decision Processes Center P50-GM68762, NCI Integrative Cancer Biology Program U54-CA112967, and NIAID R01-AI65824) and the Cambridge-MIT Institute.
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doi:10.1016/j.molimm.2007.02.008

ship between Erk activation and IL-2 production in response to peptide-MHC stimulation of TCR signaling (Kemp et al., 2007).

In the midst of increasingly intense focus on MAPK activation mechanisms (Murphy et al., 2002), the potentially independent roles of Erk1 and Erk2 in T cell signaling are open to question. Our recent data-driven statistical model determined how multiple kinase signals distal to the TCR and upstream of IL-2 promoter elements integrate to influence IL-2 production in response to peptide-MHC stimulation. This model indicated an especially strong dependence on Erk1 and Erk2 – in quantitative combination with other key kinase pathway signals such as Akt – for successful prediction of the effects of PLP peptides possessing varying TCR avidities for the 1B6 TCR (Kemp et al., 2007). The model elucidated similar correlations of Erk1 and Erk2 to IL-2 production from an experimental data compendium of 1B6 hybridoma data.

These observations concerning Erk activation and function have lead us to the hypothesis that in our 1B6 system, IL-2 production in response to TCR stimulation may be highly sensitive to total Erk1/2 activation levels but with indistinguishable contributions from Erk1 versus Erk2. Studying the independent contributions of Erk1 and Erk2 in T cell signaling poses significant technical challenges. Clearly, it is important to vary the level of each isoform independently. In addition the ability to measure a cell response at multiple cellular concentrations of active isoform is also desirable for a quantitative understanding of the contributions of that isoform. Pharmacological inhibitors have the advantage that inhibitor doses can be varied resulting in a range of active protein. One disadvantage of pharmacological inhibitors is that typically these cannot distinguish between the two isoforms because they act upon the common Erk upstream kinase, MEK. Traditional knockout experiments focusing on multiple aspects of development have been performed (Saba-El-Leil et al., 2003; Pages et al., 1999; Agrawal et al., 2006; Fischer et al., 2005). The disadvantage of these experiments is the difficulty of generating multiple levels of active protein.

Our approach takes advantage of the varying levels of efficiency in RNA interference between non-overlapping sequences for a target protein. We generated an epi-allelic series (Hemann et al., 2003) of Erk1 and Erk2 stable shRNA T cell lines that resulted in a quantitative range of respective knockdown degrees in either isoform individually. These seven knockdown cell lines enabled rigorous examination of the relationship between total Erk, phospho-Erk, and IL-2 secretion, yielding a strong quantitative relationship between Erk, phospho-Erk and IL-2 production across the range of Erk1 and Erk2 knockdown levels, with Erk1 and Erk2 effects quantitatively similar as predicted.

2. Methods

2.1. Generation of stable knockdown cell lines

Replication-deficient lentivirus was produced in 293FT packaging cells (Invitrogen) as described previously (Rubinson et al., 2003). The pLKO.1 vectors with appropriate shRNA sequences were obtained from the RNAi Consortium, as a kind gift from David E. Root (Moffat et al., 2006). Forty-eight hours after transfection, viral supernatants were collected, filtered through a 0.45 μm filter and used for infection immediately or stored at 4 °C for several days. 10^5 1B6 hybridoma cells were spin-infected with viral supernatant in 24 well-plates for 1.5 h at 2500 rpm at 30 °C in the presence of 8 μg/ml polybrene. Four hours after infection, fresh media was added and cells were grown at 37 °C for 48 h. To eliminate uninfected cells, puromycin (2.5 μg/ml) was added to the cells for additional 5 days. Cells were maintained in puromycin-containing media prior to stimulation. The entire infection process was repeated for the L144 peptide results such that independent infections for different batches of virus under different stimulation conditions are reported. One Erk2 shRNA virus did not yield viable 1B6 cells following puromycin selection during the repeat, therefore eight lines were produced for the anti-CD3 results and seven lines produced for the L144 peptide results.

2.2. Semi-quantitative Western blotting

2×10^6 cells were resuspended in 200 ml media without FBS. For anti-CD3 stimulated samples, 2 μg/ml anti-CD3 (2C11, BD Biosciences) was added for 10 min at 37 °C. Cells were then lysed and boiled for 5 min in Laemmli reducing sample buffer. Ten microlitres of each sample was load per well. For L144 stimulated samples, 5×10^6 1B6 cells were added to DAS cell (Munder et al., 2002) seeded-L144 peptide coated-six-well plates, centrifuged at 1000 rpm for 1 min, and incubated for 10 min at 37 °C and lysed as previously described (Kemp et al., 2007). Ten micrograms of protein was loaded per well. After electrophoresis, samples were transferred to polyvinylidene difluoride (PVDF) membrane and blocked with 3% BSA in TBST. Blots were visualized and quantified using a Kodak Image Station 1000. The net intensity of individual bands was determined. Anti-total and anti-phospho p44/42 (Thr202/Tyr204) antibody was from Cell Signaling Technologies, and anti-rabbit HRP from Santa Cruz Biotechnology.

To determine the level of protein knockdown for each cell line, the intensity of each Erk band was first normalized to its tubulin control. This normalization accounts for differences in protein loading from one sample to another. To account for differences in intensity across multiple membranes, both Erk bands across a single membrane are then expressed as fold change relative to the luciferase control band. The standard deviations reported are representative of both biological and experimental replicates.

2.3. IL-2 ELISA

Supernatant was collected from 100,000 1B6 cells stimulated for 4 h on DAS cell-seeded and L144 peptide-coated 24-well plates analyzed using quantitative sandwich ELISA for mouse IL-2 (BD Biosciences).

3. Results and discussion

3.1. A range of stable Erk1 and Erk2 knockdown cell lines can be used as a tuning tool for quantitatively modulating the TCR activation pathway

To determine whether total Erk levels could be modulated using established shRNA techniques, four unique sequences in Erk1 and four unique sequences in Erk2 mRNA transcripts were targeted for knockdown in a mouse 1B6 T cell hybridoma, generating an epi-allelic series for both Erk1 and Erk2. Stable cell lines were generated for each shRNA using lenti-viral infections and puromycin selection. As a non-specific control, stable cell lines were generated by infection with shRNAs targeting luciferase. The level of total protein knockdown for each sequence was determined by Western blotting for two separate infections (Fig. 1A and B). The fold change in Erk levels relative to luciferase control reported in Table 1 are averages of three experimental replicates for each infection. Normalization allows for comparison of multiple experimental replicates by expressing band intensities in knockdown cell lines as fraction of luciferase control.

A quantitative range of knockdown levels was generated for both Erk1 and Erk2 individually, as seen in Fig. 1. These results demonstrate our ability to generate a spectrum of stable knockdowns using shRNA constructs possessing diverse target sequences. The slight differences in knockdown levels seen in Fig. 1A and B are likely due to differences in virus titer during each infection.

3.2. No compensation occurs between Erk isoforms due to knockdown

To determine whether one Erk isoform was being up-regulated in the presence of an shRNA to the other Erk isoform, the level of the knockdown isoform was plotted against the total cumulative level of both Erk1 and Erk2 (Fig. 1C and D). We evaluated the degree of compensation of one isoform by another by probing for deviation from a linear relationship between total Erk1 + Erk2 and the percentage reduced of a given isoform. Complete compensation would be delineated by a horizontal line at fold change from luciferase control (Erk1 + Erk2) = 2. A lack of compensation would be delineated by a perfect line drawn from the luciferase control at the ordinate intercept to fold change from luciferase control (Erk1 + Erk2) = 1 at 100% knockdown for one isoform. Although slight differences can be noted between the anti-CD3 and peptide stimulation conditions, significant compensation between isoforms was not found in the 1B6 cell line.

3.3. IL-2 production is dependent on total Erk level

To determine whether there is a direct correlation between total Erk levels and IL-2 production in response to TCR stimulation, IL-2 production in response to an altered peptide ligand of PLP, L144, was measured. L144 peptide presentation to 1B6 T cells was previously described in (Kemp et al., 2007). IL-2 production by 1B6 Erk1/Erk2 knockdown cell lines was measured 4 h after peptide stimulation by ELISA

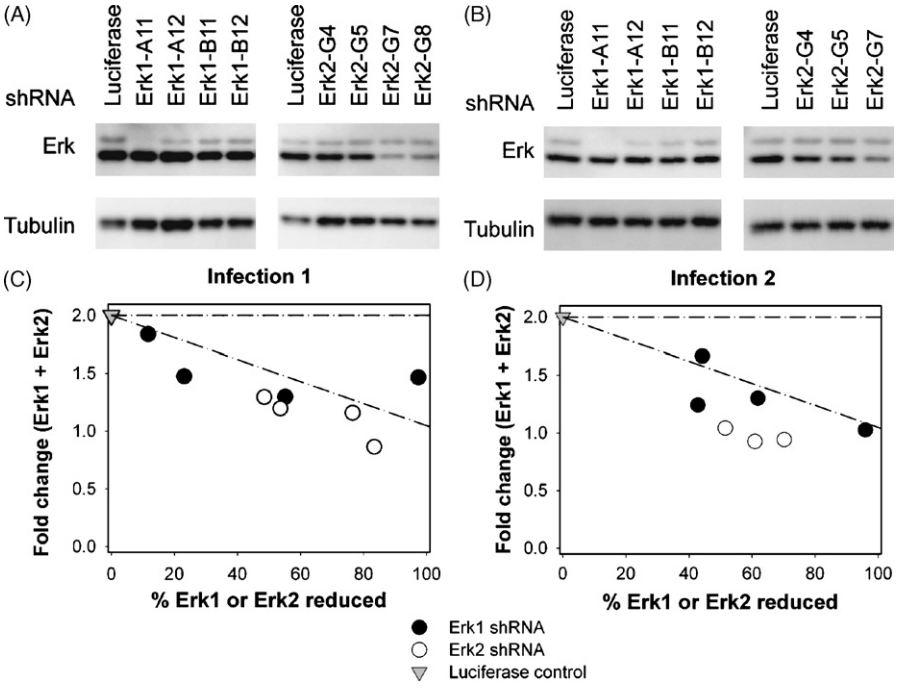

Fig. 1. Generation of a range of stable knockdown cell lines. (A and B) Total Erk Western blot of the knockdown cell lines under two separate infection (A) infection 1 and (B) infection 2. Band intensities shown are normalized as described in text. (C and D) Sum of Erk1 and Erk2 is plotted vs. the fraction of either Erk1 in an Erk1 knockdown or Erk2 in an Erk2 knockdown expressed as fold change from luciferase control for (C) infection 1 and (D) infection 2. In the case of complete compensation by one isoform for the decreased levels of another, a horizontal line at $y = 2$ is expected.

Table 1
Measured levels of total and phospho-Erk in knockdown cell lines

Cell line	Anti-CD3				L144 peptide			
	Erk1	Erk2	$Erk1_{phos}$	$Erk2_{phos}$	Erk1	Erk2	$Erk1_{phos}$	$Erk2_{phos}$
Erk1-A11	0.04 ± 0.04	0.77 ± 0.13	0.07 ± 0.06	1.19 ± 0.36	0.04 ± 0.05	0.98 ± 0.19	0.24 ± 0.08	0.79 ± 0.23
Erk1-A12	0.45 ± 0.18	0.85 ± 0.17	0.57 ± 0.28	1.58 ± 0.46	0.56 ± 0.29	1.11 ± 0.13	0.50 ± 0.13	1.05 ± 0.28
Erk1-B11	0.77 ± 0.47	0.71 ± 0.10	0.33 ± 0.22	1.15 ± 0.41	0.38 ± 0.09	0.92 ± 0.20	0.82 ± 0.13	0.97 ± 0.23
Erk1-B12	0.88 ± 0.21	0.96 ± 0.40	0.78 ± 0.24	1.29 ± 0.31	0.57 ± 0.27	0.67 ± 0.13	0.95 ± 0.50	0.83 ± 0.38
Erk2-G4	0.73 ± 0.10	0.46 ± 0.04	1.28[a]	1.01 ± 0.40	0.56 ± 0.23	0.48 ± 0.08	0.74 ± 0.63	0.53 ± 0.18
Erk2-G5	0.78 ± 0.26	0.51 ± 0.05	0.93[a]	0.40 ± 0.29	0.53 ± 0.13	0.39 ± 0.04	0.96 ± 0.58	0.46 ± 0.28
Erk2-G7	1.02 ± 0.36	0.15 ± 0.03	0.59[a]	0.31 ± 0.06	0.64 ± 0.16	0.30 ± 0.04	0.59 ± 0.24	0.35 ± 0.20
Erk2-G8	0.92 ± 0.31	0.24 ± 0.04	1.19[a]	0.40 ± 0.27	N/A	N/A	N/A	N/A

A summary of the values used in the figures ± S.D.
[a] Replicate measurements not made.

(Fig. 2C). Both Erk1 and Erk2 levels were correlated for the two infections in a similar fashion with IL-2 production for L144 peptide stimulation (Fig. 2A and B). The correlation coefficients for total levels of knockdown Erk with IL-2 production for the two infections are 0.83 and 0.93, respectively (Table 2). This suggests that IL-2 production is related to Erk levels, and demonstrates that IL-2 production can be modulated by varying the level of cellular Erk protein via either isoform.

3.4. Phospho-Erk levels are related to total Erk levels

To determine the relationship between an Erk knockdown and its associated phospho-Erk levels upon stimulation, stable knockdown cell lines were stimulated using anti-CD3, or L144 peptide loaded DAS cells and phospho-Erk levels measured via immunoblotting then normalized and depicted as fold change from luciferase control as described above (Fig. 3A and B). By comparing total-Erk levels to phospho-Erk levels in these

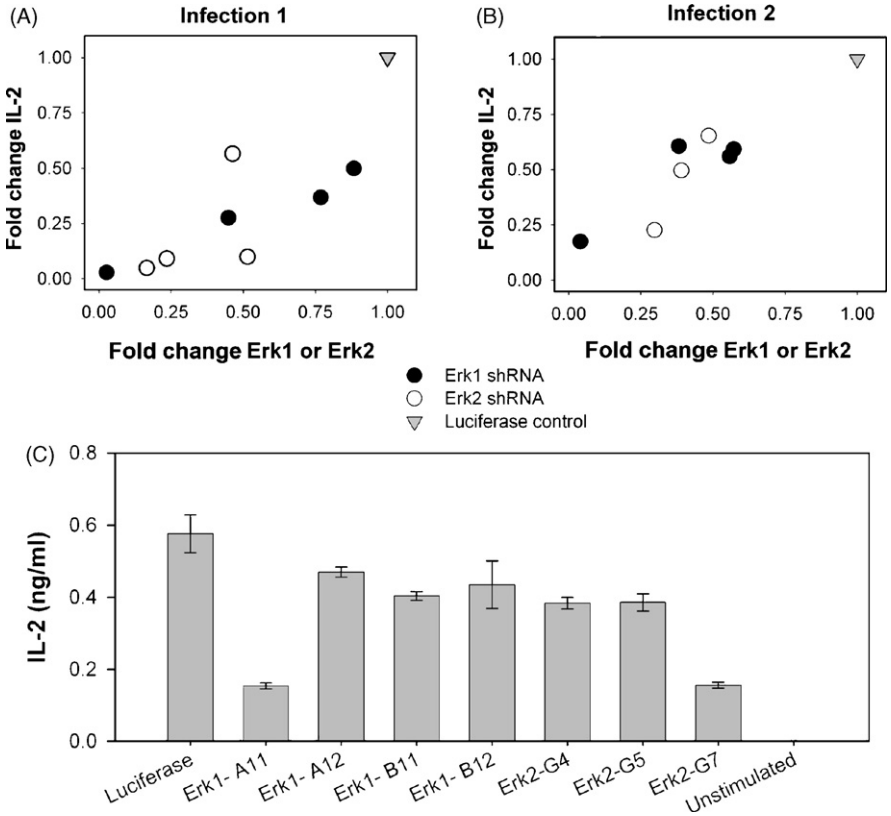

Fig. 2. IL-2 production is dependent on total Erk protein level. IL-2 levels following stimulation by for 4 h in (A) infection 1 and (B) infection 2, expressed as fold change relative to luciferase control. IL-2 levels are plotted vs. total Erk1 levels in an Erk1 knockdown or total Erk2 in an Erk2 knockdown. (C) Representative experiment of absolute values of IL-2 produced by 4 μg/ml L144 for 4 h; units are in ng/ml.

Table 2
Correlation coefficients for select plots

	Correlation coefficient
Fig. 2A	.83
Fig. 2B	.93
Fig. 3C	.69
Fig. 3D	.79
Fig. 4	.83

A summary of the correlation coefficients calculated for the relationships depicted in Figs. 2–4.

knockdown cell lines (Fig. 3C and D), we obtain a positive correlation between the two measurements (Table 2), indicating that a constant fraction of the Erk1 and Erk2 present in these cells is activated in response to TCR stimulation. The observation that phosphorylated fractions of Erk in knockdown cell lines are constant is consistent with the idea that MEK, the upstream Erk kinase, distinguishes between its substrate ERK isoforms. In addition, the amount of phosphorylation of the knockdown isoform compared to the non-targeted isoform (Fig. 3E) shows a lack a compensation at the phosphorylation level, consistent with the lack of compensation in total protein (Fig. 1C and D).

3.5. Individual Erk isoforms influence IL-2 production to similar degrees

Because phospho-Erk levels might be expected to be more proximally predictive than total Erk protein levels of IL-2 production, we tested the relationship between phospho-Erk levels

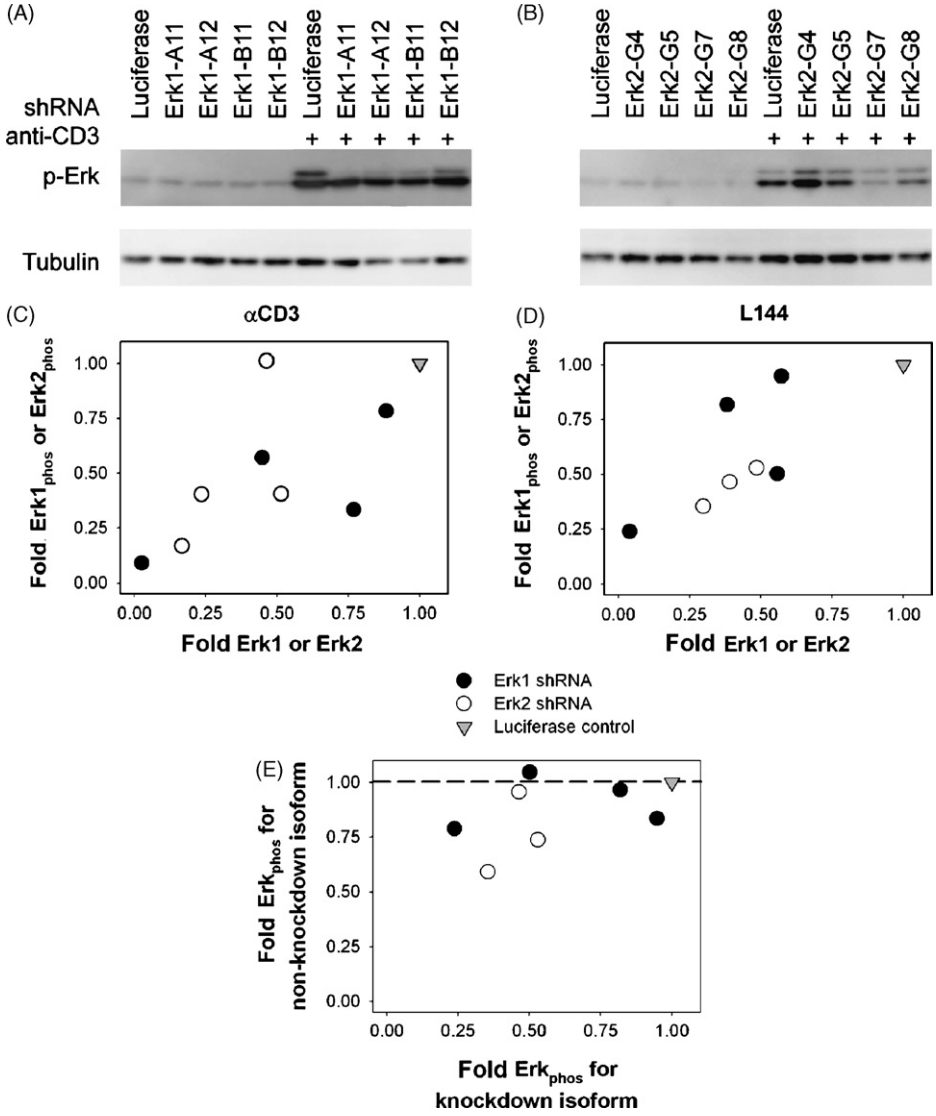

Fig. 3. Phospho-Erk levels are related to total Erk levels. (A and B) Phospho-Erk levels as measured by Western blot of the knockdown cell lines following 4 h stimulation by (A) 2 μg/ml anti-CD3 for infection 1 and (B) 4 μg/ml L144 peptide for infection 2. Band intensities shown are normalized and depicted as described in text. Standard deviations reported include both experimental and biological replicates. (C and D) Phospho-Erk1 level is plotted vs. total Erk1 level for Erk1 knockdown and phospho-Erk2 level is plotted vs. total Erk2 level for Erk2 knockdown for 4 h stimulation by (C) 2 μg/ml anti-CD3 and (D) 4 μg/ml L144 peptide. Levels are expressed as fold change with respect to luciferase control. (E) Phosphorylation levels of the knockdown Erk isoform vs. the phosphorylation levels of the untargeted isoform. In the case of complete compensation by one isoform for the decreased levels of another, a horizontal line at 1 would be expected.

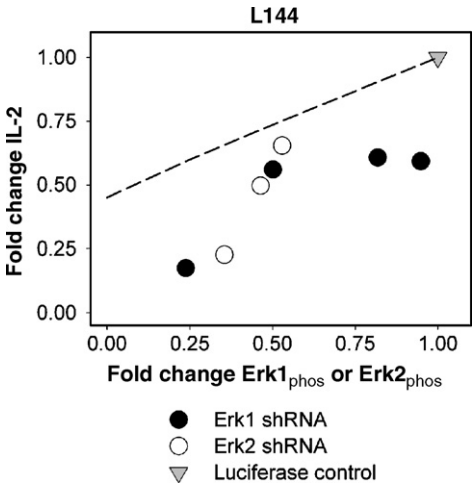

Fig. 4. IL-2 production is dependent on phospho-Erk level, with Erk1 and Erk2 yielding similar influences. IL-2 production vs. phospho-Erk1 levels in an Erk1 knockdown or phospho-Erk2 in an Erk2 knockdown are shown for 4 μg/ml L144 peptide. IL-2 measurements were made at 4 h post-stimulation and phosphorylation was quantified 10 min post-stimulation. All measurements are expressed as fold change with respect to the luciferase control. The broken line denotes the effect of phospho-Erk diminution by the upstream MEK inhibitor PD98059 as found in our previous work (Kemp et al., 2007).

and IL-2 following TCR stimulation by the L144 peptide (Fig. 4, Table 2). Similarly to the case for total Erk levels, IL-2 production appears to strongly depend on both phospho-Erk1 and Erk2 levels, indicating that IL-2 levels can be quantitatively modulated through Erk activation. Moreover, the quantitative dependencies on phospho-Erk1 and phospho-Erk2 appear to be essentially indistinguishable yet both impact IL-2 to a greater extent than would be predicted for redundant isoforms. Therefore, it is possible that each isoform is necessary and contributes uniquely to IL-2 promotion. Interestingly, Erk1 and Erk2 knockout mice exhibit different phenotypes (Saba-El-Leil et al., 2003), and conditional Erk2 knockouts have more severe T cell defects than Erk1 knockout mice (Pages et al., 1999), indicating that individual Erk isoforms might indeed be important in various cellular contexts.

Finally, the dashed line in Fig. 4 denotes the effect of phospho-Erk diminution by means of the small-molecule MEK inhibitor PD98059, as found in our earlier work (Kemp et al., 2007). Clearly, shRNA knockdown of Erk appears to lead to a greater reduction in IL-2 production than did pharmacological inhibition of the upstream kinase MEK, for equivalent degree of phospho-Erk diminution. This raises an intriguing aspect of comparing reduced protein levels to pharmacological inhibition in that Erk protein may facilitate signal transduction by more than its activity alone; the greater reduction in IL-2 for the RNAi conditions may be indicative of Erk being used as part of macromolecular complexes for other signaling reactions. Another difference between the pharmacological condition and the shRNA cell lines is the perturbation in both isoforms versus the effects of one isoform; however, reduction of the levels of both isoforms simultaneously could not be tested due to loss of cell viability for Erk1/Erk2 double knockdown cell lines.

4. Concluding note

By generating an epi-allelic series, an approach previously shown useful for determining the effect of knocking down protein levels to various extents on phenotypic outcomes (Hemann et al., 2003), we have demonstrated here that an incomplete kinase knockdown can yield information consistent with the level to which the kinase is knocked-down. It remains to be seen whether these results are upheld for different proteins in multiple systems. As ever-increasing numbers of researchers in immunology and other fields of biology rely on incomplete knockdown methods along the lines we describe here; this work may provide a helpful framework for quantitative analysis and interpretation.

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