Bypassing the need for high throughput processing in cell line development: Rapid, selective *in situ* cell screening by phosphopeptide recognition

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Aim of study

- The aim of the present study was to test if small phosphopeptides could be used as probes to rapidly and selectively screen *in situ* for cells producing antibodies against the phosphopeptide

Introduction

- Animal cell culture technology has been thrust to the forefront of the biopharmaceutical industry because of the superior fidelity of mammalian cells in producing clinically relevant proteins
- Costs for developing new therapeutic proteins and biomarkers are high and the industry is under pressure to shorten time to market
- A major bottleneck in both discovery and development of cell lines is screening large heterogeneous populations of cells to find the best cell line candidates
- ClonePix FL technology has been shown to rapidly isolate antigen-specific, IgG-secreting hybridoma clones[1], and high secreting candidate clones for cell line production[2]
- Here we present a one-step secretion assay to selectively isolate hybridomas producing phosphopeptide-specific IgG with minimal manual labour

Methods

Peptide probes

- 'NonPP': A 22 amino acid peptide
- 'PP': The same peptide synthesised with a phosphotyrosine in the middle of the peptide chain

Cells

- 'PhosCell': A stable hybridoma cell line secreting antibody that recognises the phosphorylation site of PP
- 'NonPhosCell': A stable hybridoma cell line secreting antibody that does not recognise PP or NonPP

Phosphopeptide-specific secretion assay

- Semi-solid assay medium was prepared at room temperature with the following:
  - CloneMatrix semi-solid concentrate (40mls)
  - DMEM 2X concentrate (50mls)
  - FBS (10mls)
  - Complex Initiation Factor
  - FITC-conjugated peptide (0.2µg/ml)
  - Rhodamine-conjugated phosphopeptide (0.2µg/ml)
- After thorough mixing, cells were added and gently mixed in
- The mixture was plated into 6-well plates at 2mL per well
- Plates were incubated at 37°C, 5% CO_2 for 7 days to grow discrete colonies
- Using ClonePix FL, plates were imaged by rhodamine and /or FITC
- Rhodamine-positive clones were picked (5µL vol.) into 96-well plates pre-filled with liquid medium (150µL per well)
- After 3 days further growth, conditioned media were removed for ELISA

ELISA assays

- Phosphopeptide-specific ELISA assay
  - 96-well streptavidin-coated plates were incubated with 2µg/ml biotinylated PP (or NonPP) for 1 hour
  - After washing and blocking, conditioned media samples were added for 45 min (typically diluted 1:50)
  - After washing, bound phosphopeptide-specific IgG was quantified using HRP-conjugated anti-mouse IgG (1:10,000)
- Mouse IgG ELISA assay
  - Total IgG was measured using a commercially available kit (Bethyl Laboratories)

Principle of screening by phosphopeptide recognition

- Concomitant with growth of clonal colonies in semi-solid medium, the complex initiation factor diffuses through the medium and creates a local precipitation complex in the vicinity of IgG-secreting hybrids
- Non-secreting hybrids or those secreting other immunoglobulin types will not form a capture complex
- Only those hybridomas that secrete IgG recognising the phosphopeptide will accumulate fluorescence via the fluorescently-conjugated phosphopeptide
- The principle is illustrated in Fig 1.

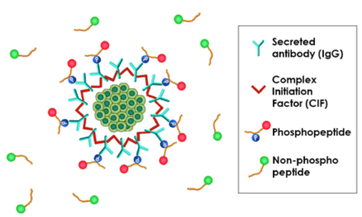

Figure 1. Principle of screening by phosphopeptide recognition

Results and Discussion

Confirmation of IgG secretion

- To first test that the two cell lines secreted IgG while in semi-solid medium, each was plated out in the presence of rhodamine-conjugated anti-IgG (CloneDetect)
- After 7 days incubation, ClonePix FL was used to image the plates by white light and rhodamine fluorescence
- Fig 2 shows that both cell lines produced discrete colonies that secreted IgG
- The test also indicated that the cell lines were heterogeneous for growth and secretion rates

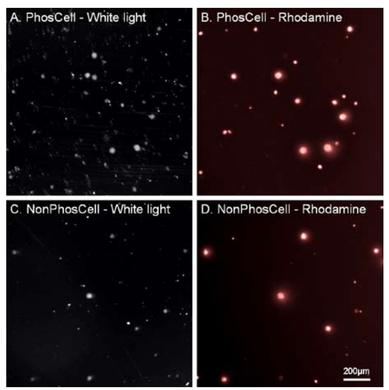

Figure 2. Confirmation that both cell lines secrete IgG. The white light images (A & C) show outgrowth of discrete colonies. The fluorescent images (B & D) show IgG secretion from viable colonies.

Selective detection by phosphopeptide recognition

- To test if ClonePix FL could detect phosphopeptide-specific clones, a 1:1 mix of the two cell lines was set up using the secretion assay described in Methods and was probed with both rhodamine-conjugated PP and FITC-conjugated NonPP
- Fig 3 shows that some of the colony outgrowths were rhodamine-fluorescent indicating the development of a local precipitation complex of secreted phosphopeptide-specific IgG and rhodamine-conjugated PP
- Fig 3 also shows that these colonies were not FITC-fluorescent, indicating specificity for the phosphorylation site on the peptide
- A parallel experiment was also set up using FITC-conjugated PP and rhodamine-conjugated NonPP. In this case, some colonies accumulated FITC but not rhodamine (data not shown)

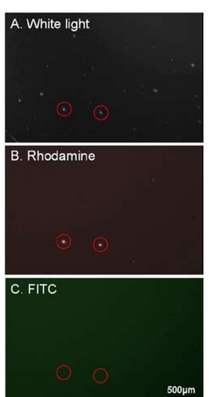

Figure 3. Selective screening of phosphopeptide-specific clones. A. White light image showing colony outgrowths. B. Rhodamine image showing some colonies accumulating rhodamine-conjugated PP. C. FITC image showing no specificity for FITC-conjugated NonPP. Typical representative colourised images are shown. Red circles in each image indicate the location of highest secreting phosphopeptide-specific clones.

Validating the phosphopeptide-specific ELISA

- To test the specificity of the clones identified by the secretion assay, a capture ELISA was established to measure phosphopeptide-specific IgG in the harvested supernatants
- Fig. 4 shows that the ELISA was quantitative for IgG secreted by the phosphopeptide-specific cell line (PhosCell + PP)
- The ELISA was unable to recognise IgG secreted by the other cell line (NonPhosCell + PP)
- The ELISA did not recognise IgG secreted by the phosphopeptide-specific cell line when the plates were coated with non phosphorylated peptide (PhosCell + NonPP)

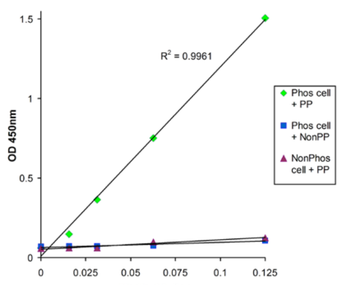

Figure 4. Dilution curve for the phosphopeptide-specific IgG capture ELISA.

Selective picking by phosphopeptide recognition

- To test the specificity of clones detected by the phosphopeptide-specific secretion assay, a 1:4 mix of PhosCell:NonPhosCell was plated into eight 6-well culture plates and probed with rhodamine-conjugated PP
- Colony outgrowths showing highest rhodamine fluorescence were picked from four plates (n=14)
- As controls, further colonies were picked randomly based on white light imaging from the other 4 plates (n=32)
- Fig. 5 shows that the secretion assay selectively isolated phosphopeptide-specific clones
- Assuming a cut off phosphopeptide IgG: total IgG ratio of 0.1 (Fig. 5A), 14 of the rhodamine positive clones (100%) and 5 of the random clones (16%) were phosphopeptide positive
- The presence of positives in the random group is consistent with the cell population being comprised of 20% PhosCell and 80% NonPhosCell
- Interestingly, total IgG shown in Fig 5C indicates that selective picking also preferentially collected the highest secreting of the phosphopeptide-specific clones

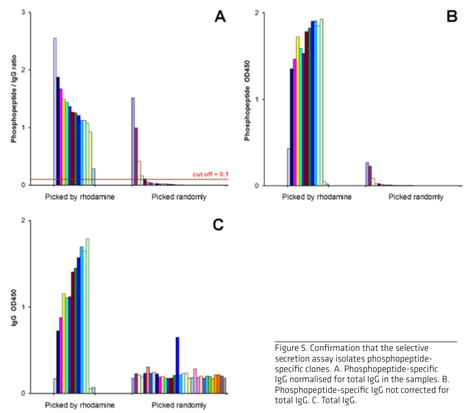

Figure 5. Confirmation that the selective secretion assay isolates phosphopeptide-specific clones. A. Phosphopeptide-specific IgG normalised for total IgG in the samples. B. Phosphopeptide-specific IgG not corrected for total IgG. C. Total IgG.

Conclusions

- This study demonstrates that clones can be selectively isolated from a mixed population based on phosphopeptide recognition
- It also demonstrates that the assay is sensitive for antigens as small as 2.6kD. We have previously shown that antigens as large as 160kD can be used[3], so the application is robust for a broad range of protein sizes
- Additionally, the assay finds the very best phosphopeptide-specific clones within populations that are believed to be clonal
- The method of *in situ* selective screening is a powerful tool for rapidly finding rare clones of interest with minimal manual input

References

1. Mann, CJ *et al.* Automated screening and selection of antibody-producing mammalian cell lines. Lab Automation meeting. San Jose, Jan 2005.
2. Watters, S *et al.* High throughput screening and selection of stable high secreting clones. ESACT UK meeting. Wyboston, Jan 2007.
3. Chuntharapai, A *et al.* Rapid selective *in situ* screening of hybridomas in semi-solid medium. SBS meeting, St Louis, Apr 2008.

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